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CHRONIC LYMPHATIC LEUKAEMIA ASSOCIATED WITH MALIGNANT DISEASE.

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THE association of malignant disease with chronic lymphatic leukaemia has been noted by many authors and has given rise to some discussion as to its significance. Videbaek (1947) has reviewed the literature in this respect but he does not confine his remarks to chronic lymphatic leukaemia. Bichel (1949) also lists the various reports which have appeared in the literature, and adds three cases of his own in which a carcinoma of the stomach was associated with chronic lymphatic leukaemia. Pathological confirmation of the carcinoma was obtained in two of the cases and was probably present in the third. He found the leukaemic process became less pronounced as the carcinoma progressed. Marischler (1896) found a similar sequence of events in a case of hypernephroma associated with lymphatic leukaemia. Penzold (1937), however, did not find this in his case of carcinoma of the stomach and lymphatic leukaemia.

Reich (1936) described a case of adenocarcinoma of the colon in which there was a characteristic blood picture of lymphatic leukaemia and the diagnosis was confirmed during life by bone-marrow examination. At post mortem, however, no evidence of leukaemia could be found. This is unusual, and in most of the reported cases there appears little doubt about the presence of both malignant disease and lymphatic leukaemia.

The frequency of the association is doubtful. Scott (1949) found one carcinoma of the breast and one carcinoma of the tongue in a series of 88 cases of chronic lymphatic leukaemia. Hoffman (quoted by Schreiner and Wehr, 1934) found one case of malignancy amongst 86 cases of leukaemia. Schreiner and Wehr (1934) considered it to be a rare association. Morrison, Feldman and Samwick (1944) found 2 cases of malignant disease amongst 600 cases of leukaemia (lymphatic and myelogenous), but they considered some cases may have escaped notice.

In a review of the 106 cases of chronic lymphatic leukaemia which have passed through the cancer clinics in Saskatchewan, Canada, since their origin in 1932, it was found that there was an associated malignant lesion in no fewer than 20 cases. In a further 3 cases there was probably an associated malignant lesion, but because of insufficient details they were excluded from this review.

A diagnosis of chronic lymphatic leukaemia was accepted if the disease lasted longer than 6 months, and if the peripheral blood-count showed grossly raised leucocytes with a preponderance of lymphocytes. The diagnosis was also accepted when the leucocyte count was moderately and persistently raised and there was a preponderance of lymphocytes. In any doubtful cases bone-marrow examination and lymph-node biopsy were carried out to establish the diagnosis. The malignant disease was confirmed histologically in all but one. The distribution was as follows : skin and lip cancer (10) ; stomach (3) ; breast (2) ; prostate (1) ; rectum (1) : bladder (1) ; lung (1) ; and melanotic sarcoma (1).

The salient features of these cases appear to be worth recording.

Case 7, a male aged 65 years, was first seen on March 4, 1948, complaining of pain in the left hypochondrium. Examination showed a generalised lymphadenopathy with the spleen enlarged a hands-breadth below the costal margin. Blood count showed Hb 86 per cent, R.B.Cs. 4,100,000, leucocytes 149,500 with 75 per cent lymphocytes. Deep X-ray therapy was given to the spleen, axillae and groins, resulting in a drop in the white cell count. The patient's condition was satisfactory until May, 1949, when rapid deterioration occurred and he succumbed on June 6, 1949.

Post-mortem examination showed a carcinoma of the stomach and leukaemic infiltration of the spleen, lymph nodes and, to a lesser extent, the liver.

Case 17, a male aged 53 years, was diagnosed as lymphatic leukaemia in 1938. His condition remained fairly satisfactory, and in April, 1943, the blood count was Hb 58 per cent R.B.Cs. 2,780,000, and the leucocytes 126,250, with 85 per cent lymphocytes and 9 per cent smudge cells. The spleen was enlarged a hands-breadth below the costal margin and there was a generalised lymphadenopathy. Deep X-ray therapy resulted in a drop in the leucocytes to 14,500. Blood transfusions were given, but a fairly severe degree of anaemia persisted. In August, 1943, the patient died following a series of haematemeses.

Post-mortem examination showed generalised involvement of the viscera with leukaemic infiltration but, in addition, an adenocarcinoma of the stomach.

Comment : In both these cases the leucocytes dropped, but this would have been expected following the course of radiotherapy. The carcinoma was an incidental discovery at post mortem in both cases.

Case 62, a male aged 72 years, was seen in December, 1945, complaining of general malaise, anorexia, loss of weight and oedema of the ankles. Physical examination showed enlarged lymph nodes in the axillae and groins and the spleen was enlarged one hands-breadth below the costal margin. Barium meal examination showed a filling defect in the region of the pylorus. Blood count was Hb 62 per cent, R.B.C.s 3,640,000, and leucocytes 295,000 with 67 per cent lymphocytes. Radiotherapy was given to the spleen and lymph nodes, but the patient's condition gradually deteriorated and he succumbed in March, 1946.

Comment : No post mortem was performed in this case, but the clinical picture and sequence of events made the diagnosis of carcinoma of stomach probable. Following radiotherapy the leucocytes dropped to 100,000 and remained at that level subsequently.

Case 28, a female aged 38 years, was first seen in August, 1949, with a 6 months' history of a lump in the right breast. On examination a firm mass could be palpated in the right breast, and a few small lymph nodes could be palpated in the neck, axillae and groins. Blood count showed Hb 91 per cent, leucocytes 39,700, with

81 per cent lymphocytes and 8 per cent polymorphs. Radical mastectomy was performed on August 19, 1949, and pathological examination of the tissue showed a spheroidal cell carcinoma. In December, 1950, the spleen was just palpable and the blood count was Hb 74 per cent, leucocytes 244,500 with 94 per cent lymphocytes. A course of deep X-ray therapy was given to the spleen and the leucocytes dropped to 92,000. The patient felt well when last seen in November, 1951.

Case 33, a female aged 75 years, was seen on January 5, 1949. She had noticed a lump in the left breast for 3 weeks. Physical examination confirmed the presence of a mass in the left breast. Enlarged lymph nodes were present in both axillae and the spleen and liver edge could be palpated. Blood count showed Hb 87 per cent, leucocytes 77,050 with 67 per cent lymphocytes. In December, 1950, a simple mastectomy was performed, and the specimen showed a spheroidal-cell carcinoma of the breast. When last seen in September, 1951, the patient was still well and the blood count showed Hb 95 per cent, leucocytes 94,500 with 95 per cent lymphocytes. No active therapy was given for the lymphatic leukaemia.

Comment : In both these cases it is not possible to say the associated carcinoma altered the course of the lymphatic leukaemia in any way.

Case 31, a male aged 63 years, was seen on September 29, 1949, with a lesion on his nose which on biopsy proved to be an epidermoid carcinoma. There were no other physical signs and blood count showed Hb 91 per cent, leucocytes 30,100 with 77 per cent lymphocytes. The patient remained well and when last seen in September, 1951, the count was Hb 104 per cent, leucocytes 63,000 with 90 per cent lymphocytes. No active treatment was given for the lymphatic leukaemia.

Case 37, a male aged 67 years, was first seen in February, 1943, with a carcinoma of the lip proved by biopsy. Blood count at that time showed Hb 98 per cent, leucocytes 16,400, with 57 per cent lymphocytes and 20 per cent smudge cells. Lymphatic leukaemia was not diagnosed until 1947, when bone-marrow examination showed the typical picture. In April, 1950, the patient was found to have an epidermoid carcinoma of the face proved by biopsy. The blood count was Hb 108 per cent, leucocytes 24,300 with 61 per cent lymphocytes. There were no other abnormal physical signs. His condition remained satisfactory, and little alteration had occurred in the blood picture when last seen in November, 1951.

Case 51, a male aged 76 years, was seen in October, 1945. His complaint was of a fungating lesion on the face. On examination there proved to be two carcinomata of the face and a further carcinoma behind the right ear. There were enlarged lymph nodes in the neck and groins and the spleen was enlarged two fingers' breadth below the costal margin. Blood count showed Hb 84 per cent, leucocytes 173,500 with 88 per cent lymphocytes. Deep X-ray therapy was given to the spleen and lymph nodes, but his condition gradually deteriorated and he died on October, 6 1946. No post mortem was performed. The leucocytes remained high and the haemoglobin gradually fell.

Case 53, a male aged 73 years, was seen in March, 1944, with an epidermoid carcinoma of the face and of the chest wall. On that occasion blood count showed Hb 95 per cent, leucocytes 6,500 with 55 per cent polymorphs and 26 per cent lymphocytes. In June, 1946, the patient found some enlarged lymph nodes in his neck and examination showed the presence of a generalised lypmhadenopathy. The spleen and liver were not palpable. Blood count showed Hb 80 per cent leucocytes 13,900, with polymorphs 4 per cent, lymphocytes 66 per cent and smudge cells 29 per cent. A node was excised and histological examination showed

it to be infiltrated with small lymphocytes. Bone-marrow examination confirmed the diagnosis. The patient remained well, but the leucocytes did not rise above 14,600.

Case 68, a male aged 68 years was seen on April 27, 1947. Twelve years previously a carcinoma of the lip had been treated and there was a local recurrence of this lesion. There were no other abnormal physical signs, but blood count showed Hb 67 per cent, leucocytes 48,700, with 70 per cent, lymphocytes and 26 per cent smudge cells. Bone-marrow examination confirmed the diagnosis. The patient was given deep X-ray therapy and was well when last seen in August, 1951, when the blood count showed Hb 66 per cent, leucocytes 71,500 with 92·5 per cent lymphocytes

Case 70, a male aged 74 years, was seen in January, 1948, for a lesion on the right temple which proved to be squamous cell carcinoma. There were many enlarged lymph nodes in the neck but the spleen and liver were not palpable. Blood count showed Hb 65 per cent, leucocytes 53,900, with 76 per cent lymphocytes and 12 per cent smudge cells. Biopsy of a lymph node confirmed the diagnosis. Deep X-ray therapy was given on 4 occasions, but the patient eventually succumbed in November, 1949. Post-mortem examination showed leukaemic invasion of the lymph nodes, bone marrow, spleen, liver, lungs, kidney and right adrenal.

Case 87, a male aged 53 years, was seen in September, 1949, with a lesion on his nose which biopsy showed to be a rodent ulcer. There were some nodes in the neck and axillae but no other abnormal physical signs. Blood count showed Hb 87 per cent, leucocytes 110,000 with 88 per cent lymphocytes. Deep X-ray therapy was given in August and September, 1951, but the leucocytes did not alter a great deal. The patient was still well when last seen in November, 1951.

Case 89, a male aged 74 years, was seen in October, 1949, with a lesion behind the left ear which proved to be an epidermoid carcinoma. There were no other signs or symptoms, but blood count showed Hb 94 per cent, leucocytes 104,000 with 90 per cent lymphocytes. No therapy was given for the leukaemia and he had no symptoms when last seen in November, 1951.

Case 97, a male aged 62 years, presented in November, 1950, with a lesion on his lip which proved to be an epidermoid carcinoma. There was a generalised lymphadenopathy but the spleen and liver were not palpable. Blood count showed Hb 100 per cent, leucocytes 56,700 with 78 per cent lymphocytes. No therapy was given for the leukaemia. The patient was well in November, 1951, when last seen, although the spleen was then palpable. The leucocytes at that time were 71,000 with 85 per cent lymphocytes.

Case 104, a male aged 63 years, was seen in September, 1949, with an epidermoid carcinoma of the face. There were no abnormal physical signs. Blood count showed Hb 91 per cent, leucocytes 30,200 with 77 per cent lymphocytes. No therapy was given for the leukaemia. He was well when last seen in November, 1951, when the blood count showed Hb 88 per cent, leucocytes 70,500 with 92 per cent lymphocytes.

Comment : In this group of skin cancers it is doubtful if the lymphatic leukaemia was influenced in any way whatsoever. In all cases the skin cancer was treated as if no leukaemia was present and a satisfactory response obtained. Case 53 was interesting in that the carcinoma appeared 2 years before the diagnosis of leukaemia was made.

Case 42, a male aged 79 years, was seen on November 30, 1948, complaining

of general malaise. Physical examination showed a generalised lymphadenopathy, and blood count was Hb 104 per cent, leucocytes 16,500, with 16 per cent polymorphs, 49 per cent lymphocytes and 33 per cent smudge cells. Sternal marrow examination showed the typical picture of lymphatic leukaemia. The patient developed urinary symptoms, and in November, 1950, transurethral resection was done and pathological examination of the tissue showed a carcinoma of the prostate. No active treatment was given for the lymphatic leukaemia. The patient was well when last seen in November, 1951.

Case 43, a female aged 60 years, was first seen in August, 1947, with a malignant melanoma of the right leg. At that time the liver edge and tip of the spleen were palpable but there were no enlarged lymph nodes. Blood count showed Hb 73 per cent, leucocytes 88,250 with 6 per cent polymorphs, 57 per cent lymphocytes and 35 per cent smudge cells. The malignant melanoma was excised and a course of deep X-ray therapy was given to the spleen. This resulted in a drop in the leucocytes, but the patient developed secondary deposits in the liver and died on September 9, 1949.

Case 61, a male aged 75 years, was seen on June 28, 1945, with a benign lesion of the face. There were some small lymph nodes in the groins and blood count showed Hb 84 per cent, leucocytes 46,600, with 7 per cent polymorphs and 93 per cent lymphocytes. In December, 1945, the patient developed rectal bleeding, and sigmoidoscopy showed a lesion of the rectum which on biopsy proved to be an adenocarcinoma. Treatment was refused, and the patient eventually developed symptoms of intestinal obstruction and succumbed in June, 1946.

Case 74, a male aged 70 years, was seen in September, 1948. He complained of painless haematuria which had been present for 4 years. Cystoscopy showed a malignant lesion of the bladder, which on biopsy was found to be a transitional cell carcinoma. Blood count was Hb 83 per cent, leucocytes 38,850 with 84 per cent lymphocytes. Bone-marrow examination confirmed the diagnosis of lymphatic leukaemia. The carcinoma was treated with radon seeds and the patient's condition was satisfactory when last seen in July, 1951. No active treatment had been given for the lymphatic leukaemia. In July, 1951, the blood count was Hb 96 per cent, leucocytes 18,000 with 89 per cent lymphocytes. The spleen and liver were not palpable, but there were some moderately enlarged lymph nodes in both groins and the left axilla.

Case 106, a male aged 56 years, was seen on January 18, 1946, with a 3 months' history of upper abdominal discomfort and general malaise and weakness. On examination there was a generalised lymphadenopathy, the spleen was enlarged a hands-breadth below the costal margin and the liver edge was palpable. Blood count showed Hb 71 per cent, R.B.Cs. 3,910,000 and leucocytes 388,000, with 5 per cent polymorphs and 95 per cent lymphocytes. A course of deep X-ray therapy resulted in a fall in the leucocytes to 90,000, but the patient's condition gradually deteriorated and he succumbed on October 10, 1946.

Post-mortem examination showed an epidermoid bronchogenic carcinoma in addition to leukaemic infiltration of the abdominal viscera.

Comment: The course of the lymphatic leukaemia in this group of cases appeared to differ in no way from those in which there was no associated cancer.

The association of 20 cases of malignant disease in a group of 106 cases of chronic lymphatic leukaemia seems to be significant, and is considerably higher than in any series hitherto reported. However, there are additional factors to be con-

sidered. The incidence of skin and lip cancer in Saskatchewan (population approximately one million) is high ; in 1949 396 new cases were seen, and in 1950, 349. This is possibly related to the long hours of bright sunshine to which the predominantly farming community is exposed. Furthermore, the average age incidence of the whole series of 106 cases was much higher than in most published accounts. The maximal age incidence was found between 60 and 75 years as compared with 45 to 60 years given by Whitby and Britton (1950) and 45 to 55 years given by Leavell (1938). This may be related to the long life that Saskatchewan people seem to have. Nevertheless it is considered probable that if a routine blood count was done more frequently in old people, particularly in those referred because of malignant disease or vague general symptoms, then more unsuspected cases of chronic lymphatic leukaemia would be discovered.

This report would not be complete without some comment on the management of the malignant disease when the patient also has chronic lymphatic leukaemia. The natural history of this disease is so variable that in old people particularly it is quite unjustifiable to withhold treatment simply because of the leukaemia. In many patients in this series the leukaemia appeared to be little more than an interesting academic association.

SUMMARY.

(1) Malignant disease has been found in 20 of 160 cases of chronic lymphatic leukaemia, a higher incidence than hitherto reported.

(2) There was no evidence to suggest that the malignant disease was influenced in any way by the leukaemia or that the leukaemia was influenced by the malignant disease.

(3) A plea is made for more frequent blood counts to be made in aged people.

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SARCOMA OF THE UTERUS.

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OUR interest in sarcoma of the uterus has been stimulated by several recent encounters with the condition. This experience has prompted us to survey and report a series of cases occurring in this hospital since 1937, following the series previously reported by Handley and Howkins (1937).

Sarcoma of the uterus has attracted the attention of both clinicians and pathologists since Mayer's report in 1861. Papers published since that date have all recorded small series of cases treated in a variety of ways ; they all make depressing reading, as the condition is notoriously difficult to diagnose clinically and carries a high mortality. We have collected 26 cases, in no way selected, occurring between the years 1937 and 1950. One case was rejected due to insufficient data, leaving 25 for study. They came predominantly from the ward practice of a large general hospital and a small associated special hospital.

It seems to us rather fruitless to try and deduce the frequency with which uterine sarcomata occur. Suffice it to say that the condition is not common (Perry, 1948), and that our 26 cases occurred in about 20,000 gynaecological admissions. Many attempts have been made, especially by American contributors, to relate the number of uterine sarcomata to the number of fibromyomata seen (Lynch and Maxwell, 1931), or to assess the relative frequency of uterine sarcoma and carcinoma (Donaldson, 1933). In some series the number of sarcomata seen is related to the total number of uterine tumours (Igna and Mitchell, 1943 ; Browne, 1950). We believe that the results of most of these mathematical exercises may be fairly summed up in the statement that sarcoma of the uterus is a rare condition. It appears to be generally accepted that not more (and probably a good deal less) than 1 per cent of uterine fibromyomata undergo sarcomatous change.

The age incidence of our cases is shown in Table I. The youngest was 39 and the oldest 72 years.

TABLE I.—*Age Incidence of Twenty-five Cases of Uterine Sarcoma.*

Age. (years.)	Number of cases.
Up to 40	5
41–50	11
51–60	5
Over 60	4

Three of these cases were single women ; owing to lack of information in several of the cases it was impossible to assess the influence of parity on the condition; We are unable to subscribe to the view that sarcoma of the uterus is considerably

more common in the post-menopausal woman. We encountered only 6 post-menopausal women in our series, all of whom presented with bleeding as the dominating symptom, as reference to Table II will show.

TABLE II.—*Symptoms of Twenty-five Cases of Uterine Sarcoma.*

Symptom.	Number of cases.
Menorrhagia and/or irregular bleeding	12
Post-menopausal bleeding	6
Abdominal enlargement	4
Abdominal pain	2
Retention of urine	1
Diarrhoea and dyspnoea	1

(One case presented with both post-menopausal bleeding and abdominal enlargement.)

These symptoms are common to most series of uterine sarcomata. The case presenting with acute retention of urine was unusual. The tumour had to be shelled out of the uterus before it could be freed from the pelvis, and a subtotal hysterectomy was performed. The patient was unfortunately lost sight of after the war, but was known to have survived the operation by 7 years.

The difficulty of correct pre-operative diagnosis is notorious, and will be referred to again. It is probably true to say that the most suggestive features of a case would be post-menopausal enlargement of a fibroid, perhaps accompanied by bleeding.

TABLE III.—*Diagnosis of Twenty-five Cases of Uterine Sarcoma.*

Diagnosis.	Number of cases.
Correct pre-operative diagnosis	2
Correct diagnosis at laparotomy	4
Curettage and subsequent histological diagnosis	5
Laboratory diagnosis after hysterectomy for "fibroids" and menorrhagia	14

It will be seen that the problem of diagnosis in these cases is one of considerable difficulty. Our experience appears to be in close agreement with that of others (Evans, 1920; Lynch and Maxwell, 1931), and it is worth noting perhaps that one of our cases correctly diagnosed pre-operatively presented with both post-menopausal bleeding and abdominal enlargement, a combination that strongly suggests uterine sarcoma. Schjött-Rivers (1949), however, makes the unusual claim that 75 per cent of uterine sarcomata can be correctly diagnosed pre-operatively, but then concedes that the *common* pre-operative diagnosis is one of fibromyomata, subsequently corrected either at laparotomy or on histological examination.

Pathology and Prognosis.

A more detailed analysis of these cases is now attempted and certain well recognised features of malignancy are considered, with a view to correlating them with prognosis.

In most cases there was only one histological section available for study and in these instances every high-power field ($\times$ 485) was examined. Where there was more than one section available, at least 50 fields were examined in each section.

It is thus apparent that some cases will have had a more detailed examination than others, so that a truer picture will be obtained in these cases.

The following features were considered :

I. Relation of tumour cells to blood-vessels.
II. Degree of cellularity.
III. Variability of cell size.
IV. Cellular morphology.
V. Presence of giant cells.
VI. Mitosis.
VII. Hyperchromatism.
VIII. Site of origin.

I. *Relation of tumour cells to vessels.*

The cases were grouped according to whether tumour cells were in contact with the blood stream. In some cases malignant cells could be seen lying in and invading preformed vessels, capillaries and veins, though no arteries were seen to be involved. In others the cells were lining blood-filled spaces with only a suggestion of a preformed vessel (Fig. 1), most easily seen in a Van Gieson stained section. In the remainder there were blood spaces lined purely by tumour cells. The cases falling into the first two categories were grouped together under the heading " Related to Blood Vessels." The results are expressed in Table IV.

TABLE IV.—*Length of Survival in Cases Related and not Related to Blood-vessels.*

Not related to blood-vessels.			Related to blood-vessels.		
14	years	A & W	18	months	D
13	,,	,,	10	,,	,,
12	,,	,,	9	,,	,,
11	,,	,,	9	,,	,,
11	,,	,,	7	,,	,,
9	,,	,,	4	,,	,,
8	,,	,,	2	,,	,,
8	,,	,,	1	,,	,,
7	,,	,,	1	,,	,,
7	,,	,,	9 cases		
3	,,	,,			
3	,,	,,			
1	,,	,,			
6	months	D			
3	,,	,,			
2	years	,,			
16 cases					

In this and subsequent tables D = Death of the patient from sarcoma of uterus. The time is that which elapsed between operation and death. A & W = Alive and well. The time is that which elapsed between operation and when last seen at follow-up clinic.

From Table IV it is seen that all the cases related to blood-vessels died within a period of 18 months following operation. In the group showing no relationship to blood vessels, 3 cases died within 2 years of operation. The remaining cases have survived up to 14 years after diagnosis up to the present time. We thus see that the relationship of tumour cells to blood-vessels distinguishes the highly malignant cases from those of lesser malignancy.

II. *Degree of cellularity.*

The degree of cellularity of a tumour is often of assistance as an aid to prognosis, since in general the more cells and the less stroma the more malignant is the tumour. In order to discover if this feature is of value in assessing prognosis in uterine sarcoma the cases were grouped as follows, according to the proportion of cells per field as compared to stroma :

Group 1 : Lesser degree of cellularity.
Group 2 : Medium degree of cellularity.
Group 3 : Greater degree of cellularity.

The findings based on the degree of cellularity of the tumour showed no correlation with length of survival so that a detailed table of the findings is not included.

III. *Variability in cell size.*

The degree of variation in cell size in the tumour was expressed as follows :—

Group 1 : Small variation in cell size.
Group 2 : Moderate variation in cell size.
Group 3 : Large variation in cell size (Fig. 2).

The results are expressed in Table V.

TABLE V.—*Length of Survival and Number of Patients in each Group.*

Group 1.		Group 2.		Group 3.	
14 years	A & W	12 years	A & W	1 year	A & W
13 ,,	,,	7 ,,	,,	10 months	D
11 ,,	,,	18 months	D	6 ,,	,,
11 ,,	,,	9 ,,	,,	2 ,,	,,
9 ,,	,,	9 ,,	,,	4 cases	
8 ,,	,,	7 ,,	,,		
8 ,,	,,	1 ,,	,,		
7 ,,	,,	7 cases			
3 ,,	,,				
3 ,,	,,				
2 ,,	D				
4 months	,,				
3 ,,	,,				
1 ,,	,,				
14 cases					

In Group 1 (showing the least variation in cell size), 4 cases out of the 14 died within 2 years of histological diagnosis. In Group 2, 5 out of the 7 cases died within 18 months of diagnosis. In Group 3 (showing greatest variation in cell size), 3 out of 4 cases died within 10 months. It is thus seen that the greater the variation in cell size, the shorter is the survival period.

IV. *Cellular morphology.*

The majority of sections show a mixed picture of round cells and spindle cells (Fig. 3). Care must be taken in deciding the shape of the cell, as a spindle cell cut transversely may be mistaken for a round cell. The best way of avoiding this

error is to determine the shape of the cell in relationship to a longitudinal section of a blood-vessel (Novak and Anderson, 1937).

The results were grouped according to the percentage of round cells and spindle cells per field. The final figure " per field " was reached by taking the average percentages after complete study of the available sections as described above :

Group 1 : Less than 32 per cent of round cells per field.
Group 2 : 33 to 65 per cent of round cells per field.
Group 3 : 66 per cent and over round cells per field.

The percentage of spindle cells per field were grouped in a similar manner.

Tabulated results show that the percentage of spindle cells in each group and their survival period are practically the exact opposite of that found in the case of round cells. Therefore, a detailed table is shown for round cells only (Table VI).

TABLE VI.

Group 1.			Group 2.			Group 3.	
14 years	A & W	.	2 years	D	.	10 months	D
13 ,,	,,	.	9 months	,,	.	2 ,,	,,
12 ,,	,,	.	9 ,,	,,	.	1 ,,	,,
11 ,,	,,	.	6 ,,	,,	.	—	
11 ,,	,,	.	4 ,,	,,	.	3 cases	
9 ,,	,,	.	—				
8 ,,	,,	.	5 cases				
8 ,,	,,						
7 ,,	,,						
7 ,,	,,						
3 ,,	,,						
3 ,,	,,						
1 ,,	,,						
18 months	D						
7 ,,	,,						
3 ,,	,,						
1 ,,	,,						
—							
17 cases							

In Group 1, 4 of the 17 cases died within 18 months of diagnosis. In Group 2, all 5 cases were dead within 2 years, 3 of them within 9 months of diagnosis. In Group 3, all cases were dead within 10 months of diagnosis. The greater the proportion of round cells, the worse the prognosis, and conversely, the more spindle cells the better the prognosis.

V. *Presence of giant cells.*

All giant cells seen were taken into account in the final result, including multinucleated ones and cells with giant nuclei. Very few giant cells showed mitotic nuclei. The cases were grouped as follows :

Group 1 : 0 to 32 per cent of cells per field that were giant cells.
Group 2 : 33 to 65 per cent of cells per field that were giant cells.
Group 3 : 66 per cent and over of cells per field that were giant cells.

The results based on the number of giant cells present showed no correlation with the length of survival.

VI. *Mitosis.*

The sections were examined and the number of all mitoses per field determined and grouped as follows :

Group 1 : Less than 1 mitosis per 5 fields.
Group 2 : More than 1 mitosis per 5 fields and less than 2 mitoses per field.
Group 3 : 2 or more mitoses per field.

The range was 1 mitosis in 30 fields up to 8 per field. The results are expressed in Table VII.

TABLE VII.—*Length of Survival and Number of Cases in each Group.*

Group 1.		Group 2.		Group 3.	
14 years	A & W	11 years	A & W	2 months	D
13 ,,	,,	7 ,,	,,	1 ,,	,,
12 ,,	,,	1 ,,	,,	1 ,,	,,
11 ,,	,,	2 ,,	D		
9 ,,	,,	18 months	,,	3 cases	
8 ,,	,,	10 ,,	,,		
8 ,,	,,	9 ,,	,,		
7 ,,	,,	9 ,,	,,		
3 ,,	,,	7 ,,	,,		
3 ,,	,,	6 ,,	,,		
		4 ,,	,,		
10 cases		3 ,,	,,		
		12 cases			

In Group 1 all cases were alive and well when last seen, up to 14 years after diagnosis. In Group 2, 9 cases out of the 12 have died within 2 years of diagnosis. In Group 3, all 3 cases died within 2 months of diagnosis. These results are in accordance with other workers (Evans, 1920), showing that the greater the number of mitoses, the worse is the prognosis.

VII. *Hyperchromatism.*

The sections were examined as stated in the introductory paragraph, and the number of hyperchromatic nuclei per field recorded.

Group 1 : 0 to 32 per cent of nuclei per field showing hyperchromatism.
Group 2 : 33 to 65 per cent of nuclei per field showing hyperchromatism. (Fig. 4).
Group 3 : 66 per cent and over of nuclei per field showing hyperchromatism.

TABLE VIII.—*Length of Survival and Number of Cases in each Group.*

Group 1.		Group 2.		Group 3.	
14 years	A & W	8 years	A & W	10 months	D
13 ,,	,,	7 ,,	,,	9 ,,	,,
12 ,,	,,	7 ,,	,,	4 ,,	,,
11 ,,	,,	1 ,,	,,	2 ,,	,,
11 ,,	,,	18 months	D		
9 ,,	,,	9 ,,	,,	4 cases	
8 ,,	,,	7 ,,	,,		
3 ,,	,,	6 ,,	,,		
3 ,,	,,	3 ,,	,,		
2 ,,	D	1 ,,	,,		
		1 ,,	,,		
10 cases		11 cases			

In Group 1 (the least number of hyperchromatic nuclei), 1 case out of the 10 died within 2 years of diagnosis. In Group 2 (intermediate group), 7 out of the 11 cases died within 18 months of diagnosis. In Group 3 (the highest number of hyperchromatic nuclei), all 4 cases died within 10 months of diagnosis. It is thus seen that the higher the proportion of hyperchromatic nuclei, the shorter becomes the survival period.

VIII. *Site of origin.*

Table IX shows the distribution of cases as to their site of origin.

TABLE IX.

Site of origin.	Number of cases.
Leiomyoma	20
Endometrium	1 definite 4 " probables "
Myometrium	0
Blood-vessels	0

The majority of cases arose on a fibroid basis (Fig. 5). In only 1 case was it thought that the tumour had definitely arisen outside a fibroid, from the endometrium. Macroscopically this tumour replaced the entire endometrium and was of soft and bulky consistency, with infiltration into the myometrium in some areas. Microscopically there was no evidence of any previous fibroid basis for the tumour. The tumour was highly cellular, with a minimum amount of stroma. The case showed blood-vessel invasion, and death occurred within 9 months of diagnosis. The 4 " probable " cases arising from the endometrium showed no related fibroid, but the picture in some areas suggested that they *may* have arisen from a fibroid.

It is of interest to record that 3 out of these 4 cases died within 1 month, 2 months and 10 months of diagnosis ; the fourth case was alive and well when last seen, 9 years after the initial diagnosis.

Treatment.

Both surgery and radiotherapy as the basic form of treatment have their advocates. and there are those who place their faith in some combination of the two (Crossen and Crossen, 1941 ; Cooke, 1943 ; White, 1949).

The published series of cases are all small, and it is difficult to get any impression of the efficacy of treatment from a study of the figures. As we see it, the problem is twofold :

I. The treatment to be adopted when the diagnosis is made pre-operatively or at operation.

II. Treatment of those cases in whom the diagnosis has been made in the laboratory :

(*a*) Following complete uterine removal.

(*b*) Following myomectomy or subtotal hysterectomy.

1. In those cases where the correct pre-operative diagnosis has been made, radical hysterectomy is probably the treatment of choice (Browne, 1950). The problem at issue in these cases is whether pre- and/or post-operative deep X-ray therapy should be employed Opinions differ markedly on the point. Perry (1948) maintains that deep X-ray therapy is of little value, and is supported in this view

by Bosse and Stanton (1943). At the Radiumhemmet, Heyman (1950) does not consider that preliminary radiotherapy is indicated, and only advises post-operative irradiation when sarcomatous tissue has been found in the edge of the removed specimen. Randall (1943) believes that in cases of endometrial sarcoma diagnosed on curettage, preliminary intracavity radium followed by radical surgery may be the treatment of choice. The type of hysterectomy performed is probably not important, but most authorities advocate a wide pelvic clearance, omitting, however, glandular dissection. It is a matter for regret that a correct pre-operative diagnosis is seldom made. Our findings indicate, however, that the prognosis depends far more upon the degree of malignancy of the growth than upon the nature of the treatment.

II. The more difficult, though numerically more numerous, group is that in which the diagnosis is made in the pathological laboratory.

If initial surgical removal has been complete, there is nothing further that can be done, apart from a course of deep X-rays of doubtful value, whatever the nature of the growth. If, however, the removal has been incomplete (subtotal hysterectomy or myomectomy), further surgical treatment might be planned, bearing in mind the accurate prognosis that we have shown can be provided by complete pathological assessment. It is along these lines we suggest that the prognosis will be improved in this condition.

TABLE X.—*Treatment and Survival.*

Treatment.	Number of cases.	Alive.	Died.
Complete removal of uterus with or without adnexal removal	12	7 (from 1–14 years after operation)	5 (from 1–18 months after operation)
Complete removal and deep X-ray	2	1 (9 years after operation)	1 (within 2 years)
Subtotal hysterectomy alone	5	3 (from 7–12 years after operation)	2 (from 4–9 months after operation)
Subtotal hysterectomy and deep X-ray	5	2 (11 years after operation)	3 (between 1 and 10 months after operation)
Deep X-ray only	1	0	1 (within 3 months)

Complications and Spread.

In those of our cases examined post-mortem, local recurrences, peritoneal spread, pulmonary and cerebral metastases have been found. We have not encountered deposits in bones, as recorded by Brooke and Thomason (1945), or in the kidneys and intestines (Browne, 1950). Regardless of the method of treatment, 9 of our 26 cases (36 per cent) were dead within a year, and a further 3 within 2 years. Ten of our cases are known to have survived 5 years (40 per cent), a figure that compares favourably with those reported in the literature (Kimbrough, 1934; Novak and Anderson, 1937; Randall, 1943). Cooke (1943) was only able to report 10 to 14 per cent five-year survival.

DISCUSSION.

It will be seen from our results that a classification of uterine sarcomata based on the relationship of tumour to blood-vessels distinguishes the highly malignant

cases from those of lesser malignancy. Every case showing such a relationship died within a period of 18 months following diagnosis.

The degree of variation in cell size bears a definite relationship to survival time : the greater the variation, the worse the prognosis. This feature is difficult to evaluate numerically, so that the results will tend to vary with the individual observer. This detracts from its value, but nevertheless from the comparison of a number of cases one can gain a definite impression of the degree of variation in cell size which is of prognostic significance.

Cellular morphology is of value in assessing malignancy. Our results show that the greater the proportion of round cells to spindle cells the worse the prognosis. It would appear, from our results, that more than 33 per cent of round cells per field carries a much graver prognosis than a value below this figure. All our cases with more than 33 per cent of round cells per field died within a period of 2 years following diagnosis ; indeed all but one case died within 10 months. The corollary holds for spindle cells, as the higher the proportion of spindle cells, the better the prognosis. These results are in accordance with those of other workers (Kimbrough, 1934).

The degree of cellularity of uterine sarcoma has proved to be of no value in assessing prognosis. This point is easily understood, as the proportion of cells to stroma is extremely difficult to assess with any accuracy.

The presence of giant cells in these tumours showed no correlation with survival time. We agree with Evans (1920) that giant cells are more likely to be associated with degeneration than with the rate of growth. This is borne out by the fact that very few of these giant-cell nuclei show mitotic figures.

The number of mitoses present serves as a sound prognostic index. Our results show that less than 1 mitosis per 5 fields carries a good prognosis ; all in this group were alive and well from 3 to 14 years following diagnosis, this being the range of their follow-up period. In those exhibiting more than 1 mitosis per 5 fields and less than 2 mitoses per field, 75 per cent died within 2 years of diagnosis and all with more than 2 mitoses per field died within 2 months of diagnosis. It would appear that there is a " critical level " of 1 mitosis in 5 fields. The greater the number of mitoses per field above this level the worse the prognosis. Our results bear out the excellent work done by Evans (1920) on mitotic counts, though we have used a simpler estimation, obviating the counting of mitotic nuclei per cubic millimetre. It should be noted that all mitoses were counted, whether normal or abnormal (Fig. 4).

Nuclear hyperchromatism is of value in assessing prognosis. The higher the percentage of hyperchromatic nuclei the worse the prognosis. All our cases with more than 66 per cent of hyperchromatic nuclei per field died within 10 months of diagnosis, while only 1 with less than 32 per cent died during the follow-up period.

As regards the site of origin of these tumours, our figures suggest that those of endometrial origin carry a worse prognosis than those arising in a previous leiomyoma, but our cases of extra fibroid origin are too few to warrant a definite conclusion. These results are in agreement with those of previous workers. There is often considerable difficulty in determining the exact site of origin, as a rapidly growing sarcoma arising in a fibroid may destroy the original pattern and lead one to a mistaken diagnosis of " myometrial sarcoma " or " endometrial sarcoma".

In the present state of our knowledge the rational treatment of this condition would seem to be radical surgical removal. Both pre- and post-operative X-ray

therapy are of doubtful value only. In those cases in which the removal has not been radical (myomectomy or subtotal hysterectomy), we suggest that further treatment should be based upon a careful assessment of the prognosis by the method discussed above. It is only by such an approach to the problem that an occasional further case can be saved.

SUMMARY.

Twenty-five cases of uterine sarcoma occurring in a twenty-year period are presented, and the following histological features are found to be of value in assessing prognosis :

(1) Relation of tumour to blood vessels.
(2) Variation in cell size.
(3) Cellular morphology.
(4) Number of mitoses;
(5) Nuclear hyperchromatism.
(6) Site of origin of the tumours.

The degree of cellularity and the percentage of giant cells were of no value in assessing prognosis.

If the diagnosis is suspected either before or during operation, radical pelvic clearance would seem to offer the best prognosis, although it must be realised that the ultimate chance of survival rests upon the degree of malignancy as demonstrated by careful pathological assessment.

If the diagnosis is unsuspected and the surgery has been incomplete, we feel that further treatment should be based on a careful consideration of the histological nature of the growth and the prognosis resulting from this.

Our thanks are due to the consultant gynaecological staff of the Middlesex Hospital and the Hospital for Women, Soho, under whose care these cases were treated, for permission to record them, to Professor R. W. Scarff for access to the material, and to Professor J. Gough for much helpful advice and criticism.

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EXPLANATION OF PLATE.

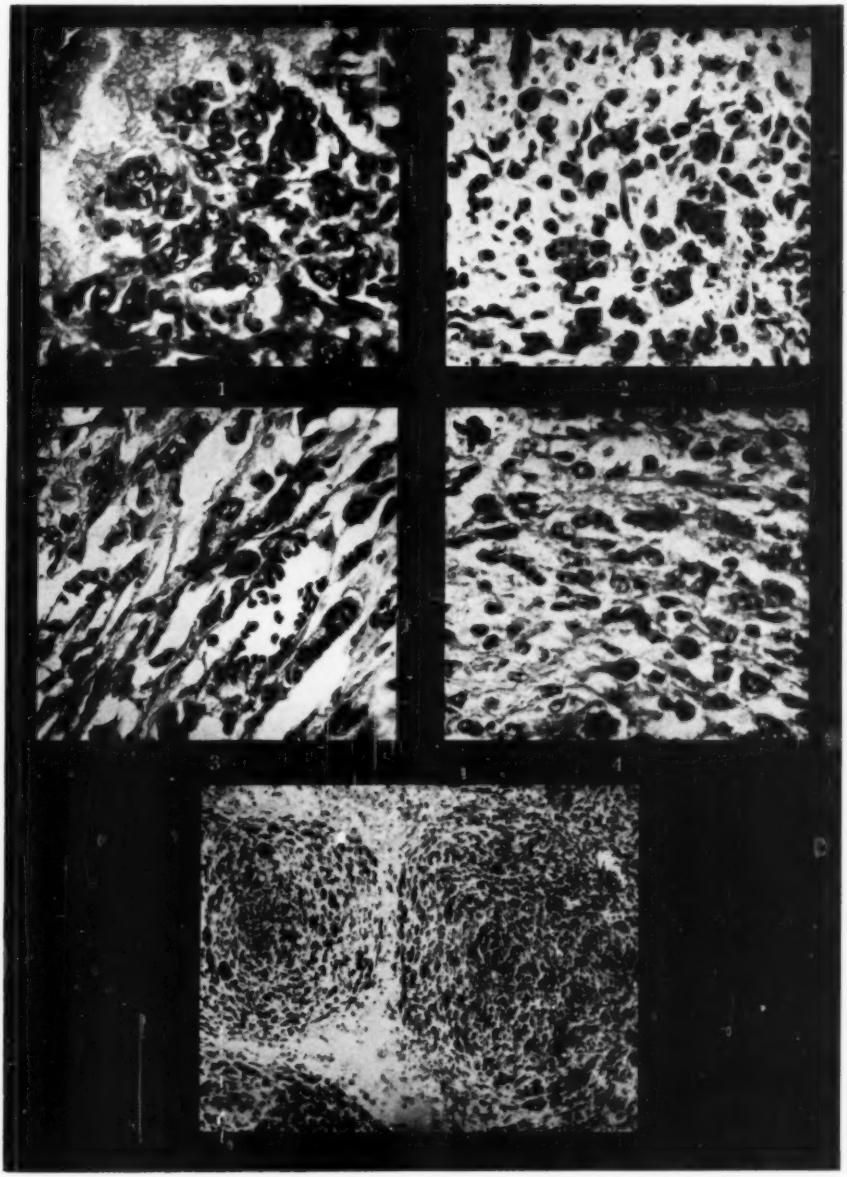
Fig. 1.—Tumour cells can be seen lining a blood-filled space. It is not certain whether this represents a preformed blood-vessel. H. and E. × 350.

Fig. 2.—Large variation in cell size. Group 3. H. and E. × 300.

Fig. 3.—A mixture of round and spindle cells. From a case classified as Group 1, with less than 32 per cent round cells. H. and E. × 370.

Fig. 4.—Two abnormal mitoses (Group 3) and 33 to 65 per cent of hyperchromatic nuclei (Group 2). H. and E. × 340.

Fig. 5.—" Whorling," suggesting sarcomatous origin in a pre-existing fibroid. H. and E. × 75.



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THE PATHOLOGY OF EPITHELIAL CYSTS AND TUMOURS IN THE JAWS.

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LESIONS of the jaws composed of, or containing, epithelium not infrequently present difficulties in their histological interpretation. While a full understanding of the nature of these lesions requires a knowledge of oral embryology and some familiarity with dental surgery, the general pathologist can readily deal with the majority of specimens likely to come to his notice, if he is aware of the diagnostic possibilities and pitfalls. From this point of view, then, epithelial lesions of the jaws are conveniently classified on a morphological basis.

I. *Periapical granuloma* containing epithelium.

II. *Simple cysts* :—

i. *Lined by squamous epithelium* :
- *a*. Dental cyst.
- *b*. Dentigerous cyst
- *c*. Eruption cyst.
- *d*. Primordial cyst.

ii. *Lined by columnar epithelium* :
- *a*. Developmental inclusion cysts.
- *b*. Dental cysts, rarely.

The cysts of this group may also be lined by squamous epithelium.

III. *Neoplasms* :—

i. *Epithelial only* :
- *a*. Adamantinoma.
- *b*. Primary squamous cell carcinoma.
- *c*. Basal cell carcinoma.
- *d*. Mucous gland tumour.
- *e*. Metastatic carcinoma.

ii. *Mixed tumours* :
- i. Adamantinofibroma.
- ii. Complex composite odontome.

Pathogenesis.

Periapical granuloma.—When chronic infection occurs at the root of a tooth there is absorption of the alveolar bone with the formation of an area of granulation tissue. This constitutes the periapical granuloma. Normally present around the roots of the teeth are small accumulations of cells, the epithelial rests of Malassez, remnants of the enamel organ. Very often the presence of infection stimulates these rests to proliferate, so that strands of epithelium grow into the

granulation tissue. In this way many granulomata come to show the presence of epithelium.

Dental cyst.—As the epithelial strands penetrate through the granuloma areas of granulation tissue become encircled by loops of epithelium, and it is thought that in some cases degeneration of these enclaves forms the starting point of the dental cyst. The initial microcysts grow larger and coalesce, so that in time a sizeable cyst is formed.

Another view holds that cyst formation starts initially in the epithelium. As the epithelial rests increase in size the central cells become in time so far placed from their source of nourishment that degeneration ensues.

In many cases the cause of cyst formation is the propensity of epithelium to cover a denuded area. Chronic abscess formation is frequent in periapical infection, and the resulting cavity may become lined by epithelium.

Dentigerous cyst.—The dentigerous cyst is derived from the enamel organ. In Fig. 1, which shows a tooth germ at an early stage of development, the enamel organ is the cap-shaped structure, consisting of a layer of columnar or cubical cells enclosing a stellate reticulum. It surmounts the dentine papilla, an area of primitive connective tissue. Enamel is deposited by the ameloblasts, the cells lining the inner surface of the enamel organ, while the dentine is laid down in apposition to the enamel by cells of the dentine papilla. As amelogenesis proceeds the enamel organ diminishes in size to accommodate the growing amount of enamel. This diminution occurs at the expense of the stellate reticulum, which ultimately disappears. The inner and outer layers of the lining cells therefore finally meet.

In certain cases the enamel organ becomes cystic. The tooth therefore becomes surmounted by, and partially contained in, a cyst, and is thus prevented from erupting. Such a cyst, in which the crown of a tooth projects into the cavity, is a dentigerous cyst.

Eruption cyst.—This is a small cyst which forms above a tooth about to erupt. It is derived from the enamel organ, or possibly from epithelial rests.

Primordial cyst.—The primordial cyst is also derived from the enamel organ, though the exact nature of its formation is obscure.

Developmental cysts.—The developmental cysts occur in relation to the embryonic lines of fusion of the face and mouth. During the process of fusion oral epithelium or vestigeal epithelial structures may be included, so that cysts may form in the mid-line of the palate or the alveolar processes, in the incisive canal, at the junction of the globular and maxillary processes, or at the junction of the globular, lateral nasal and maxillary processes.

Adamantinoma.—The pathogenesis of this tumour has given rise to much discussion, though it is generally accepted that it originates from some part of the epithelial component of the odontogenic apparatus. Lucas and Thackray (1951*a*) give reasons for believing the usual origin to be from the epithelial rests of Malassez.

Squamous cell carcinoma.—Squamous cell carcinoma may originate within the jaw, as a central tumour, or may invade the jaw from elsewhere.

The central tumour has two possible sources of origin. It may arise by squamous metaplasia in an adamantinoma, or from epithelial inclusions in the substance of the jaw—probably the rests of Serre, according to Willis (1948). These rests are the remains of the dental lamina, the bridge of epithelium which

connects the enamel organ to the oral ectoderm. The lamina breaks up at an early stage of development and almost entirely disappears, leaving only the rests.

Squamous cell carcinoma originating from without includes tumours invading the jaw by direct extension from the gum, the tongue or the palate, and metastatic deposits.

Basal cell carcinoma.—A number of authors consider the adamantinoma to be essentially a basal cell carcinoma. Certainly some adamantinomata show areas very similar to what would normally be called basal cell carcinoma, and conversely, basal cell tumours in other parts of the body may show a marked resemblance to adamantinoma. However, here we are concerned with basal cell carcinoma of the jaw as a distinct entity. Such tumours are rare, and appear most probably to originate from epithelial rests, or from the surface epithelium.

Mucous gland tumour.—Tumours resembling those of the salivary glands may occur within the jaw. They are presumed to originate from epithelial rests or from aberrant glandular tissue.

Metastatic carcinoma is relatively rare in the jaws ; cases of secondary deposits from primary growths in the lip, prostate, breast, kidney, thyroid, stomach, bronchus have been described (Stones, 1951).

The mixed tumours.—These tumours originate from both elements of the odontogenic apparatus, ectodermal and mesodermal.

Morbid Anatomy and Histology.

The form in which the pathologist receives material depends largely on the clinical features of the lesion and the stage of treatment. Thus in the case of a periapical granuloma surgical treatment may take the form of extraction of the tooth or of apicectomy. In the former case the specimen consists of a tooth with a soft, velvety red mass, some 1 to 3 mm. in diameter, attached to the apex. In other cases the operation of apicectomy is performed, so that the specimen, consists of the apex of a tooth with the granuloma adherent to it. Or the specimen may consist simply of curettings. Histologically the material consists of granulation tissue permeated by strands of squamous epithelium.

If actual cyst formation has taken place, the treatment consists of complete enucleation of the cyst if small or of moderate size. With large cysts the procedure of marsupialization is often carried out, in which a window is opened into the cyst and an obturator fitted. Gradually decreasing sizes of obturator are used, the cyst slowly becoming obliterated.

Where the cyst has been removed entire, it is usually found to consist of a round or ovoid unilocular sac, the majority of specimens measuring from a small fraction of an inch up to $\frac{3}{4}$ in. in their long axes. The cyst wall is occasionally very thin ; more often it is in the region of $\frac{1}{16}$ in. thick. The contents may consist of fairly thin fluid, often with the shimmering appearance imparted by the presence of cholesterol, but usually they consist of thicker material of pultaceous appearance. In the case of the marsupialized cyst the specimen consists of the portion of cyst wall removed at operation. It is of no very characteristic appearance.

Histological examination shows the cyst wall to be composed of connective tissue with a lining of squamous epithelium, often incomplete and rarely kera-

tinised. There is practically always heavy chronic inflammatory infiltration, and a characteristic finding is the presence of cholesterol clefts (Fig. 2). These may be situated in the necrotic material in the cyst cavity or they may be present in the connective-tissue wall, in which case foreign body giant cells are usually present. Occasionally small islands of epithelium are present in the cyst wall, at some distance from the lining (Fig. 3, 4, 5, 6).

Dental cysts may rarely be lined by columnar epithelium of sinus origin, if situated in the maxilla.

The treatment of the dentigerous cyst is carried out on similar lines to that of the dental cyst. The nature of the cyst will be obvious to the pathologist only if it is removed complete with a tooth or teeth *in situ*. In all other respects it resembles the dental cyst. The question of neoplasia occurring in the lining of cysts is dealt with later.

The remaining cysts of the jaws are usually small and removed complete. To the pathologist they present as small unilocular cysts, lined by squamous or columnar epithelium. There are no special features except that the incisive canal cyst often contains mucous glands in the connective tissue of its wall.

From the above it will be gathered that treatment of these cysts proceeds according to clinical indications. The specimens which reach the pathologist are, as it were, the by-products of treatment, in much the same way as is the appendix, removed for acute appendicitis and examined as a confirmatory measure. In the case of tumours of the jaw, however, the procedure is often different. Clinical suspicion that a lesion may be neoplastic usually leads to biopsy, treatment being deferred till the pathological examination is made, though from time to time the clinical and radiological appearances are considered to be sufficiently characteristic by themselves. In such cases the pathologist receives a portion of resected jaw as his first specimen.

The adamantinoma occurs typically as a multilocular cyst. The loculi contain a clear, yellowish fluid, and though the lining epithelium is often smooth, in some tumours there are numerous papillary projections which may even fill up the cavities, producing a solid appearance. The biopsy specimen is thus likely to consist of a portion of cyst lining, or of a small piece of solid tissue.

The microscopic appearances of the adamantinoma are well known, the tumour consisting essentially of follicles of epithelial cells in a connective-tissue stroma. The follicles bear a considerable resemblance to the normal enamel organ, consisting, like that structure, of a layer of columnar or cubical cells surrounding a central area in which the cells have a stellate appearance (Fig. 7 and 8). Histological variations are often seen (Robinson, 1937 ; Thoma, 1950 ; Lucas and Thackray, 1951*a*).

Squamous cell growths may present difficulties. The frank squamous-cell carcinoma occurring as an extension from a growth of a nearby epithelial surface or as a secondary deposit presents the usual appearances. Squamous metaplasia occurring in an adamantinoma, however, is sometimes a misinterpretation of certain other appearances. Cystic degeneration in adamantinoma is common, and sometimes the degenerating cells become swollen and homogeneous, the cytoplasm staining bright red with eosin. The appearances then resemble squamous metaplasia and have been so described and figured, but they are essentially degenerative (Fig. 9). Genuine squamous metaplasia is probably quite rare.

Tumours of the purely basal cell type are very uncommon. When they do occur they appear as adenocystic growths of familiar type. As mentioned above, areas of adamantinomata sometimes present similarities to basal cell growths, though in these cases definitely adamantinomatous tissue is also present (Fig. 10).

Mucous gland tumours occurring within the jaw are confusing mainly because of their unusual situation, and have been diagnosed as atypical adamantinomata.

Metastatic deposits of carcinoma present no unusual features.

The mixed tumours likely to be encountered by the general pathologist are the adamantinofibroma and the complex composite odontome. The adamantinofibroma, or soft mixed odontome, presents clinically as a gradually enlarging tumour of the jaw. Radiologically there is an area of translucency, often in association with an unerupted tooth. The clinical diagnosis may therefore be dentigerous cyst, unilocular adamantinoma, or fibrosarcoma in the more rapidly growing types. Histologically, the tumour consists of strands and groups of epithelial cells in a fibroblastic matrix. The epithelial cells may be cubical, or there may be differentiation towards a columnar form, while the mesodermal element of the tumour is composed of fibroblasts which produce varying amounts of collagen (Fig. 11). Sometimes the tumour is included as a type of adamantinoma. Malignant change has been described.

The complex composite odontome, like the adamantinofibroma, arises from both components of the odontogenic tissues. It presents as a smooth hard swelling of the jaw, usually in early life, and often in association with a missing tooth. The diagnosis is usually made on clinical and radiological grounds, but occasionally the tumour may be removed as a possible osteoma or osteosarcoma. Microscopically there is an irregular conglomeration of all the tooth-forming tissues (Fig. 12). Enamel epithelium may be prominent in early specimens.

EXPLANATION OF PLATES.

FIG. 1.—Early tooth germ. The cap-shaped enamel organ consists of a stellate reticulum bounded by a continuous layer of tall and cubical cells. The cells lining the deep aspect are those which form enamel. The whole structure surmounts the dentine papilla. × 20.

FIG. 2.—Dental cyst. The lining consists of squamous epithelium. Numerous cholesterol clefts are present. × 35.

FIG. 3.—Islands of epithelium in the connective-tissue wall of a dental cyst. × 35.

FIG. 4.—Detail from Fig. 3. × 150.

FIG. 5.—Another example of epithelial islets in a cyst wall. × 35.

FIG. 6.—Detail from Fig. 5. × 150.

FIG. 7.—A typical adamantinoma.

FIG. 8.—Higher magnification of an adamantinoma follicle. The basal layer of cells resemble ameloblasts; the inner cells are similar to the stellate reticulum of the enamel organ. × 150.

FIG. 9.—An adamantinoma follicle showing degenerative changes simulating squamous metaplasia. × 150.

FIG. 10.—An area from an adamantinoma showing a similar appearance to basal-cell carcinoma. × 35.

FIG. 11.—Adamantinofibroma.

FIG. 12.—Complex composite odontome, showing an irregular conglomeration of dental tissues.

FIG. 13.—An epithelial outgrowth from the lining of a dentigerous cyst. × 35.

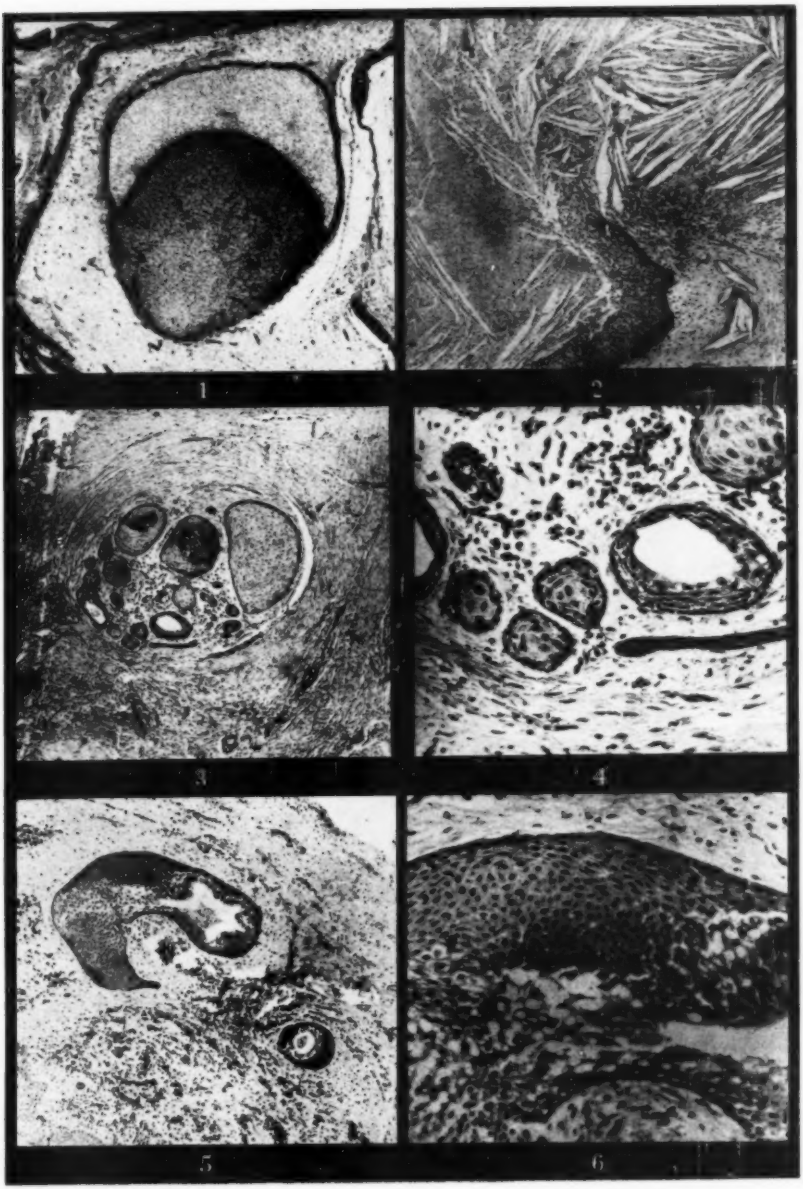
FIG. 14.—Adamantinoma forming anastomising strands of epithelium. × 100.

FIG. 15.—Anastomosing strands of epithelium in the wall of a dentigerous cyst. × 100.

FIG. 16.—Another example of epithelial proliferation in the wall of a dentigerous cyst. × 35.

FIG. 17.—Proliferating, non-neoplastic epithelium forming islets with pseudo-stellate appearance. × 35.

FIG. 18.—Detail from Fig. 11. × 150.



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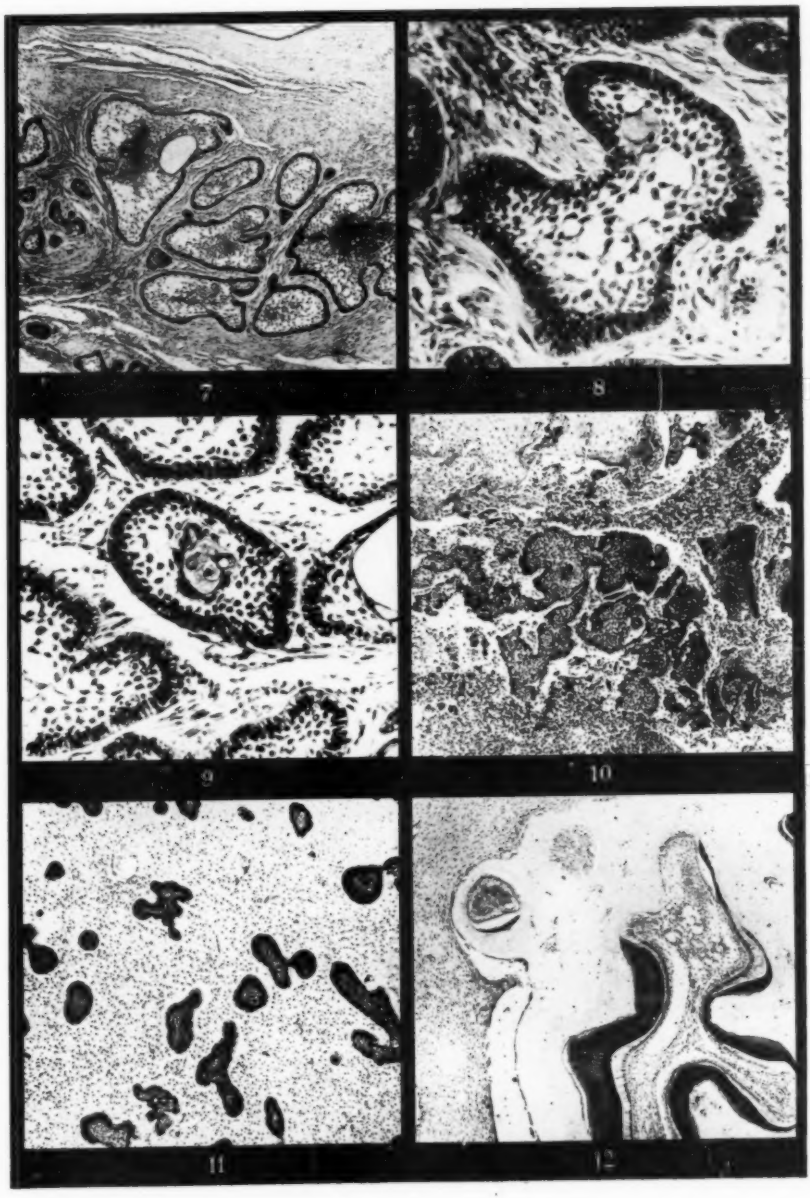

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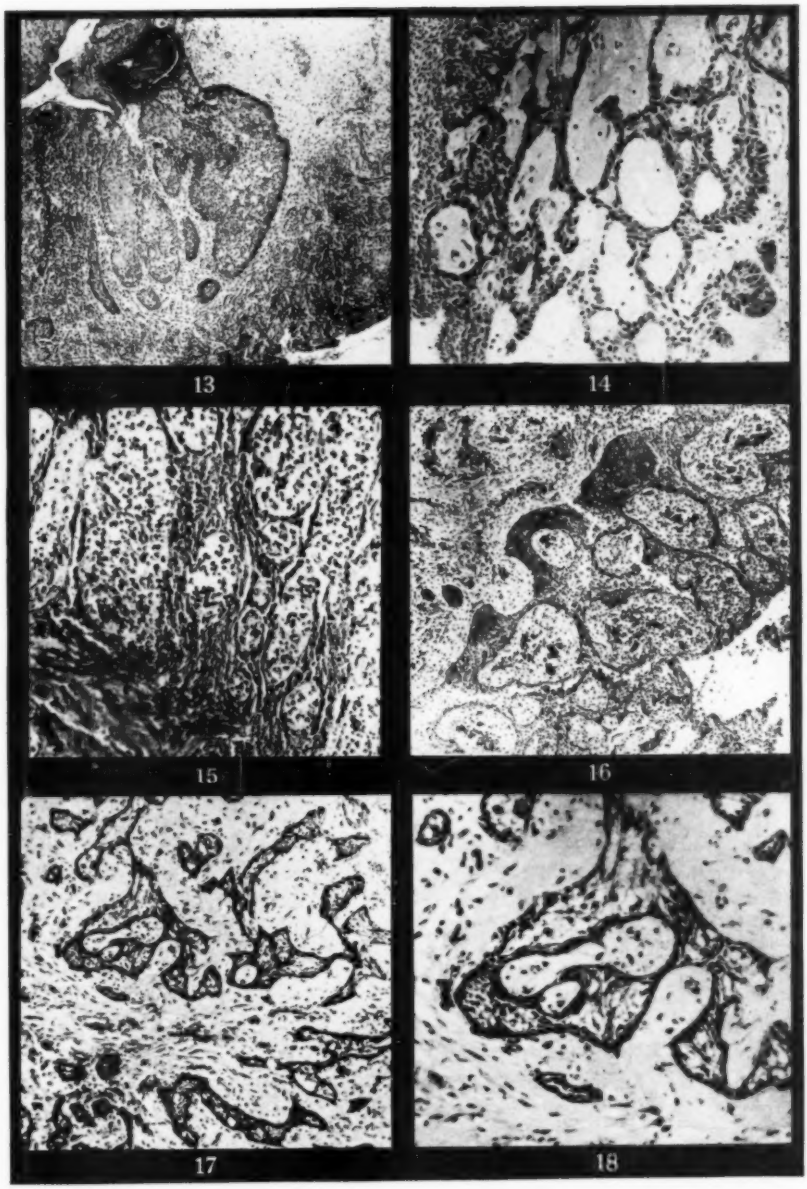

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Lucas.

The question of neoplastic change supervening in simple cysts must also be considered. The development of adamantinoma from the lining of dentigerous cysts has been reported by several authors (Cahn, 1933 ; Thoma and Proctor, 1937 ; Cameron, 1951, and others), but this occurrence is probably very uncommon. The pathologist should remember its possibility, but undue emphasis should not be placed on small areas of proliferation in a cyst lining which otherwise shows the characteristics of non-neoplastic epithelium. An example of such proliferation is shown in Fig. 13, in which an epithelial outgrowth bears some resemblance to an adamantinomatous follicle. Similarly, small islands of epithelium in the connective tissue of cyst walls, such as those seen in Fig. 3 and 5, have on occasion been thought to represent the beginnings of neoplasia.

Not uncommonly the cysts in adamantinomata occur in the stroma as well as in the epithelial masses. Sometimes they are almost entirely stromal (Lucas and Thacray, 1951*b*). The resulting appearance may be misinterpretated when only small biopsy specimens are available for examination. Thus in Fig. 14 the epithelium of the tumour tends to form anastomosing strands rather than discrete follicles, with cystic changes in the stroma, and the picture may be confused with the proliferating, but non-neoplastic epithelial strands in the wall of a dentigerous cyst (Fig. 15 and 16).

Willis (1948) believes that " adamantinomatous " appearances in squamous epithelium are not uncommon in various sites. He holds that the so-called adamantinoma of the tibia is merely a squamous-cell carcinoma in which changes in the epithelium produce a superficial resemblance to the authentic neoplasm. Similar appearances are demonstrated in Fig. 17 and 18. These show proliferation of the oral mucosa overlying a mucous gland tumour of the palate. There is no actual tumour tissue in the field shown, but appearances such as these in biopsy specimens from the oral region may cause confusion.

In cases such as those described errors may be avoided by careful examination. Though proliferating epithelium may take up a follicular appearance, the differentiation of cells is not so distinct as in adamantinoma. In the tumour follicle the outer layer of cubical or columnar cells is well marked ; even in relatively primitive types of tumour an area showing some attempt at differentiation can usually be found. In proliferating, non-neoplastic islets of epithelium, on the other hand, the peripheral cells are never columnar, and very seldom even approach the cubical. They do, however, often show up very distinctly ; either the outer two or three layers of cells become compressed so that the darkly staining nuclei come to lie very close to each other, as seen in Fig. 3 and 13, or else, as in Fig. 17, well-marked central degeneration causes the peripheral cells to stand out in contrast.

The stellate reticulum of the adamantinoma follicle is often closely simulated by degenerative changes in non-neoplastic epithelium. The cells of stellate reticulum, however, have rather plump, vesicular nuclei, whereas the cells producing a pseudo-stellate appearance are in the process of degeneration and often show pyknotic nuclei.

The great majority of cases will cause little difficulty if the points mentioned are borne in mind, though, as always, the borderline case can give trouble, particularly when it presents in the form of a small biopsy specimen. A request for further, adequate, material is then indicated.

SUMMARY.

An account is given of those lesions of the jaws in which epithelial proliferation may occur, and some of the difficulties attending histological interpretation are discussed.

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THE FREQUENCY AND SIGNIFICANCE OF MUCIN IN SWEAT GLAND TUMOURS.

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IN an earlier paper (Lennox, Pearse and Richards, 1952) a group of 11 mucin-containing skin tumours was described. It was shown that all these tumours were of sweat gland origin, and it was suggested that (with the interesting but in practice unimportant exception of the occurrence of small amounts in occasional tumours of the rodent group) a primary skin tumour with mucin in it is always of sweat gland origin. The series then described was selected on a basis of mucin content, and could not be used as evidence of the frequency of the occurrence of mucin in an unselected series of hidradenomata, though it seemed likely that this frequency would be high. The present paper describes the findings in such an unselected series.

We have been able to add 4 further cases to the list of published mucin-containing skin tumours in our earlier paper. Gross (1907) describes a vulval hidradenoma with goblet cells and mucin. Nicholson (1923) illustrates a " basal cell carcinoma with goblet cells " of the chin similar to our Fig. 6. Sikl (1932) describes 2 " benign epitheliomata with goblet cells " of the back, both of which we would regard as hidradenomata with prominent myo-epithelium, and one of which also resembles our Fig. 6.

MATERIAL AND METHODS.

A further 82 cases of sweat gland tumour were added to the original 11. These 82 cases were wholly unselected in that they included every tumour of sweat gland origin reaching us during the relevant period of which we were able to obtain sufficient material for additional stains. The many pathologists to whom we are indebted for cases are named in the acknowledgments. Our largest single source was the London Hospital, from which we obtained 49 cases. One group of 9 vulval tumours has formed the subject of a previous publication (Deacon and Taylor, 1952), the remainder are unpublished cases. With very few exceptions, the sources of the 82 new cases are entirely different from those of the original 11. A few cases were sent to us for diagnosis, but most of our externally derived cases reached us already diagnosed as sweat gland tumours : of the latter we rejected only a very few as cystic rodents or as merely dilated sweat glands. As we do not wish to enter on a necessarily long discussion of the histological diagnosis of hidradenomata, we think it important to produce this evidence that our criteria do not differ widely from those of other pathologists.

New sections were cut in every case and stained by the trichrome-periodic-

acid-Schiff method (Pearse, 1950). More detailed histochemical studies were done on a representative selection by methods described in the earlier paper. As the results of these merely confirm without significantly extending the results of our earlier series we do not intend to describe them further. In extension of the studies of one of us on melanin in skin tumours (Lennox, 1949), Masson silver stains were done in all cases ; no melanin was found in any tumour. The observations on sweat glands of animals were based on a preliminary examination of material mostly supplied by the Hannah Dairy Research Institute. The animals examined were : Rhesus monkey, Pata monkey, brown woolly monkey, antelope, Nigerian sheep, Niceian goat, Barbary lamb, llama, cow, greyhound, grey squirrel, civet, beaver, wallaby, leopard cat, lioness, newt, frog, goldfish.

RESULTS.

Mucin in the substantial amounts seen in most of our original series was seen in 14 of our 82 new cases (17·1 per cent). Smaller amounts of mucin were seen in a further 55 cases, so that altogether 69 (84·1 per cent) of these cases contained some mucin.

Hidradenomata vary considerably in histology, but mucin appeared to be very generally distributed through the varieties. A detailed statistical analysis relating quantities of mucin to a large number of histological features and to such matters as age, sex and site was almost wholly barren. Nevertheless, though quantitative results were slight, there were some obvious qualitative differences in the mode of occurrence of mucin in different varieties of hidradenomata.

Analysis of our series showed that two very closely defined groups, and one less well defined, could be separated off from the residue. The well-defined groups were the superficial (21 cases) and the vulval (15 cases) : the ill-defined group comprised the carcinomata (6 cases). The 51 remaining cases were benign tumours of the secretory part of the gland. Among this exceedingly pleomorphic residual group three varieties of histological structure are especially common, the mixed salivary variety (16 cases), the solid variety (11 cases) and the predominantly myo-epithelial variety (9 cases). In typical cases these patterns are highly distinctive, but mixed and intermediate forms occur, and the variants are probably of much less importance than the histological individuality of the type forms would suggest.

Superficial hidradenomata.

These tumours have been well described by Gates, Warren and Warvi (1943) : they are probably what most pathologists in this country mean by the term "naevus syringo-cystadenomatosus papilliferus." The actual tumour, the papillary cystadenoma of the ducts, is formed almost invariably by a two-layered columnar epithelium which usually secretes a little mucin (in 14 out of our 21 examples), but which rarely (in 2 only of the 14) secretes large amounts. In most cases (Fig. 1) the part of the gland deep to the tumour dilates and undergoes a variety of metaplastic changes, often but not always coming to resemble an apocrine type of gland, and usually contains strongly PAS-positive mucin (it was present in 11 of the 15 cases in which the underlying sweat glands were demonstrable in the material available, including all of the cases in which metaplasia was marked). Identification of this material as mucin is based on the following characters : (*a*)

It is periodic-acid-Schiff-positive, diastase-fast; (*b*) it is peracetic-acid-Schiff negative (eliminating unsaturated lipids); (*c*) it does not stain with Sudan Black B (eliminating most phospholipids); (*d*) it is variably metachromatic with toluidine blue (indicating, in conjunction with the first three tests, the presence of acid mucopolysaccharides).

Vulval hidradenomata.

All our 15 vulval tumours (Fig. 2) secreted a moderate amount of mucin. One, in addition, contained areas in which the myo-epithelial layer had proliferated to produce localised masses of swollen eosinophile cells with strongly PAS-positive cytoplasm (Fig. 3).

Hidradenocarcinomata.

Our series includes 6 cases in which evidence of local invasiveness led to a diagnosis of malignancy. The group is obviously not homogeneous. Two are spheroidal cell carcinomata, indistinguishable from those of the breast (one malignant enough to lead to amputation), and one is a mucoid carcinoma (Case XI of our earlier paper (Lennox, Pearse and Richards, 1952)) again like a breast tumour. The others are more obviously simple degenerations of ordinarily benign varieties of hidradenoma. Two of the 6 contained mucin (cf. Stout and Cooley's 1951 series).

Deep hidradenomata.

Mixed salivary variety.—This always contains metachromatic material, often with cartilage, in its stroma, and in all but 2 of our cases contains PAS-positive mucin in its duct or cyst lumina (Fig. 4). Study of this larger series (16 cases in all) confirms the conclusion of our earlier paper that this is a variant of the hidradenoma.

Solid variety.—The curious regular pattern of the solid hidradenoma is highly characteristic but difficult to define. It seems probable that its cell groups are made up of an outer layer, one or two cells thick, of small cells of myo-epithelial origin, and a central solid core of larger cells derived from the secretory layer but quite inactive. When pure, no mucin is secreted, but this type is hardly ever really pure; parts may become cystic (Fig. 5), and elsewhere the central core becomes luminated, and the cells lining the lumen may vary considerably in type. Mucin usually appears in these variant areas. So common is this that 8 of our 11 solid hidradenomata contained mucin in some part of the tumour (large amounts in one case), but, let us repeat, where the pattern is pure no mucin is secreted.

Myo-epitheliomatous variety.—The pattern of a hidradenoma depends very largely on the balance between proliferation of the myo-epithelial and secretory layers. The former may very largely predominate, and when it does so often consists chiefly of large clear cells. The justification for referring to such a tumour as a myo-epithelioma (Sheldon, 1941; Lever and Castleman, 1952) seems slight; this is merely one variant, rarely pure, among many. The secretory layer is often deficient over wide areas, but in none of our 9 cases was it wholly absent. Some mucin was being produced somewhere in every case. The curious appearance first noticed in Case III of our earlier series was seen in 3 more cases—the secre-

tory layer was represented by large bloated cells filled with a material which stains weakly with ordinary dyes but is intensely PAS-positive (Fig. 6).

Deep hidradenomata : adenomatous pattern.—An ordinary adenomatous pattern of any purity is relatively rare, but the 2 tumours in our series which most closely approached it contained large amounts of mucin (Fig. 7).

DISCUSSION.

On the whole our observations seem to amount to this : the epithelium derived from any part of a sweat gland may secrete mucin when it forms a component of a tumour, but large amounts of it are most often produced when the predominant element in the tumour is the inner secretory layer of the deep part of the gland. Under normal circumstances the ordinary eccrine sweat gland produces no mucin, and one would not therefore expect to find any in its tumours. In our earlier paper we pointed to the breast and ovary as evidence that this anomaly is not unique, but new information about the variations of the sweat gland in other animals suggests another line of explanation.

Human temperature regulation is more exact than that of most animals, and the very efficient sweating mechanism which is its main buffer is a more nearly unique one than we generally realise. Horses sweat to cool themselves, though their sweat, unlike that of man, contains protein. No other animal has been proved to cool itself in this way—there is dispute over some species (Findlay, 1950 ; Schmidt-Nielsen and Schmidt-Nielsen, 1952), but at least it is possible to rule out abundant sweating in all species in which it has been studied. Most animals have paw-pad sweat glands which have a lubricant function. But apart from this most mammals (the rodents are the chief exception) have numerous sweat glands over the general body surface which do not in the ordinary sense of the word produce sweat. Their purpose in fact remains a mystery. We have been unable to find any studies on the histochemistry of their secretions, but we have so far been able to establish that in the ungulates (especially the cow) the material appears to be a mucoprotein (Fig. 8).

One must it seems regard the apparently simple human eccrine gland as a highly specialised and unique structure of very late evolutionary development. Just as its partner the hair is one fruit of the adaptation of the reptilian scale to an infinity of uses, so the skin gland of the mammal has been adapted to a great variety of purposes. One may trace its ancestry to the unicellular mucin-secreting glands of fish epidermis and the tubular mucin-secreting glands of the wet-skinned amphibia. Man in himself exhibits several of the potential forms of these glands—the apocrine glands, the ceruminous glands, the eyelid glands of Moll, the breast are all undoubted modifications of the same pattern. The special glands of the skunk and the civet are extreme examples of the biochemical abilities of these glands. The glands which keep moist the black parts of the dog's nose are unmistakably sweat glands, but secrete a thin mucin (Fig. 9). According to von Schumacher (1918) the hippopotamus foetus has skin glands which secrete mucin. We have, then, a picture of a gland type with a capacity for ready evolutionary adaptation, derived ultimately from a mucin-secreting gland, which is capable, with only relatively minor histological change, of producing in different species or in different sites on the same species (or even to some extent in individuals of one species—how else can each dog have its own smell ?) a series of secretions of the greatest chemical diversity. The apparent simplification to produce

what is in effect nearly pure water in man is one of its most remarkable specialisations. What the exact mechanism of the appearance of mucin in hidradenomata may be remains of course uncertain : to call it atavistic hardly goes further than to call it a name. Possibly the emergence of mutant phenotypes in a genetically unstable stock deprived of an environment capable of stabilizing it affords a parallel. At least one can say that the phenomenon we are describing becomes less surprising when one finds that the sweat gland under the stimulus of tumour growth is to some extent recapitulating part of its long and colourful evolutionary history.

One other more doubtful point is worth making. We have listed already the structures in man—the ceruminous glands, the glands of Moll and the breast which are generally accepted as sweat gland homologues. One may speculate on the possibility of a similar origin for the lachrymal glands and Bartholin's gland. With even less certainty of truth but even more interest, one might suggest the salivary glands as further homologues. Both probably are derived from the mucous glands of the surface of amphibians, and both are forms of adaptation to terrestrial life. The parotid and the buccal glands arise from the ectodermal infolding of the stomodaeum, and there is no great difficulty in regarding these as homologues of the sweat glands. The submaxillary, sublingual and palatal glands are endodermal, but modern views on embryogenesis would not regard this as such an obstacle as might appear at first sight. An interesting point in this connection is that the skin just inside the nostril, which in man has hairs but no glands, in the dog has mucin glands of structure very like that of the salivary glands. This concept of the similar evolutionary origin of the sweat and salivary glands, speculative though it may be, does appear to afford a satisfactory explanation of the similarity of some of their tumours.

Sikl (1932), whose paper came to light after this discussion had been written, has anticipated us in pointing out the relevance of the mucin secretion of skin glands in lower vertebrates. He is unwilling to regard his own two tumours as sweat gland in origin, as they do not correspond to descriptions with which he is familiar, and comes to no certain conclusions as to their origin except that they are atavistic.

SUMMARY.

1. In a series of 82 hidradenomata, 69 (84 per cent) contained mucin and 14 of these (17 per cent) contained large amounts of mucin.

2. Quantitatively there was little difference between the histological varieties of hidradenomata in the amount of mucin they produced but some qualitative differences are described.

3. In explanation of the presence of mucin in these tumours the great variation in the chemical natures of the products of the sweat glands and their homologues and in particular the presence of mucin in the secretion of the sweat glands of ungulates and in the dog's nose are discussed ; the possible common origin of sweat glands and salivary glands from the mucin-secreting ectodermal glands of amphibia is also mentioned.

Our thanks are especially due to Dr. A. W. Landells for collecting for us and Professor Dorothy Russell for allowing us to use the most valuable series of cases from the London Hospital ; we also thank Professor H. M. Turnbull for the index system which enabled the accurate location of so many old cases. We must also

thank Dr. C. W. Taylor for 10 vulval tumours, and for smaller numbers of cases Dr. D. F. Barrowcliff, Dr. C. D. Cruikshank, Dr. P. Kidd, Dr. K. J. Randall, Dr. R. A. Salm, Dr. H. G. H. Richards, and the donors of the original 11 cases acknowledged in our earlier paper. Detailed thanks to others who have helped in various ways with the biological background would be impossible; we must mention Dr. J. F. Findlay and Miss Myffanwy Goodall of the Hannah Dairy Research Institute, Ayr, Mr. E. C. Appleby and Mr. S. H. Benson (respectively of the Edinburgh and Glasgow Zoological Societies), Professor P. B. Medawar and Sir Arthur Keith. The sections are the work of Mr. J. Griffin, and the photomicrographs of Mr. E. V. Willmott.

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EXPLANATION OF PLATES.

FIG. 1.—Superficial hidradenoma, with polypoid extrusion of the tumour. No mucin in the tumour in this case, but much PAS-positive material in the dilated glands below the tumour. Periodic acid—Schiff and orange G. × 6.

FIG. 2.—Vulval hidradenoma. To show the characteristic compressed anastomosing papillary pattern, and the crenated edge of the epithelium. Small amounts of pale-staining mucin lie in the clefts. H. & E. × 120.

FIG. 3.—Vulval hidradenoma. Two-layered epithelium, with the inner layer debased and in this area partly split off from a thick outer layer of bloated granular PAS-positive cells. Periodic acid—Schiff and orange G. × 155.

FIG. 4.—Hidradenoma of the mixed-salivary variety. Ducts and clefts lined by two-layer epithelium; cartilaginous stroma; mucin in the ducts. H. & E. × 115.

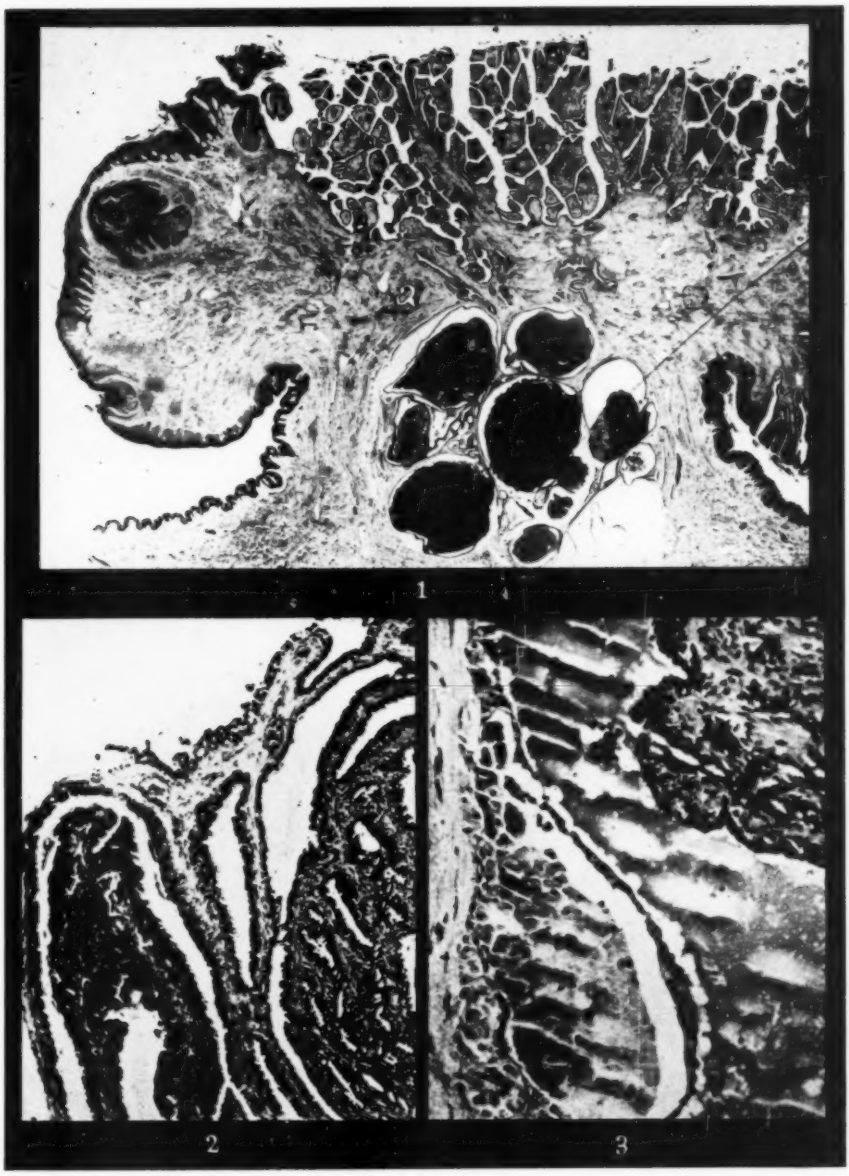
FIG. 5.—Hidradenoma of the solid variety, with transitions to an adenoid structure. The cell groups have ill-defined cores of larger paler cells which here and there show small lumina, which occasionally become small cysts. H. & E. × 165.

FIG. 6.—Hidradenoma with prominent myoepithelium. The myoepithelium forms a thick layer, often of large clear cells; on its surface the secretory layer forms a discontinuous row of bloated cells packed with deeply PAS-positive mucin (c.f. Fig. 4 of Lennox, Pearse and Richards, 1952, showing similar cells stained H. & E.). Periodic acid—Schiff and orange G. × 125.

FIG. 7.—Hidradenoma showing adenoid pattern. Two-layered epithelium and a heavy mucin secretion. H. & E. × 115.

FIG. 8.—Sweat glands of cow (Ayrshire). Showing dilated saccules of the secretory part of the gland, filled with strongly PAS-positive material. Periodic acid—Schiff and orange G. × 95.

FIG. 9.—Sweat glands of the black moist area of the nose of a dog (greyhound). Showing coil glands resembling apocrine glands, secreting mucin. Periodic acid—Schiff and orange G. × 280.



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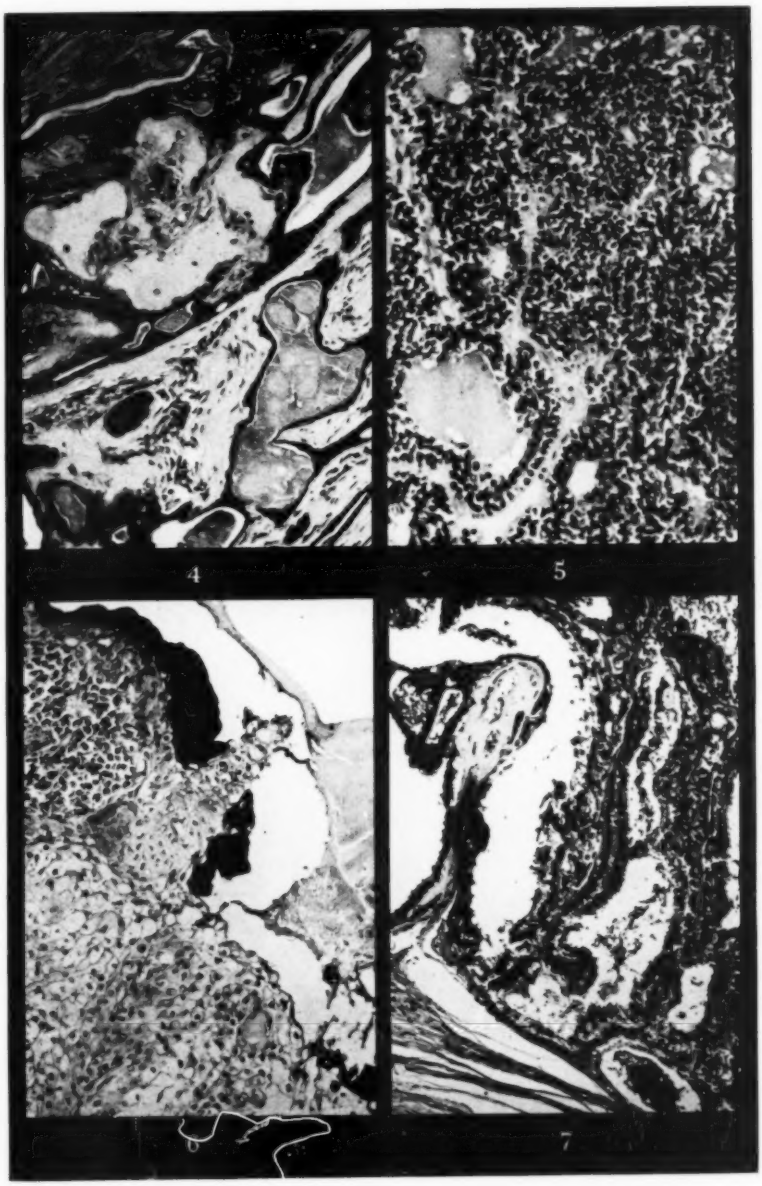

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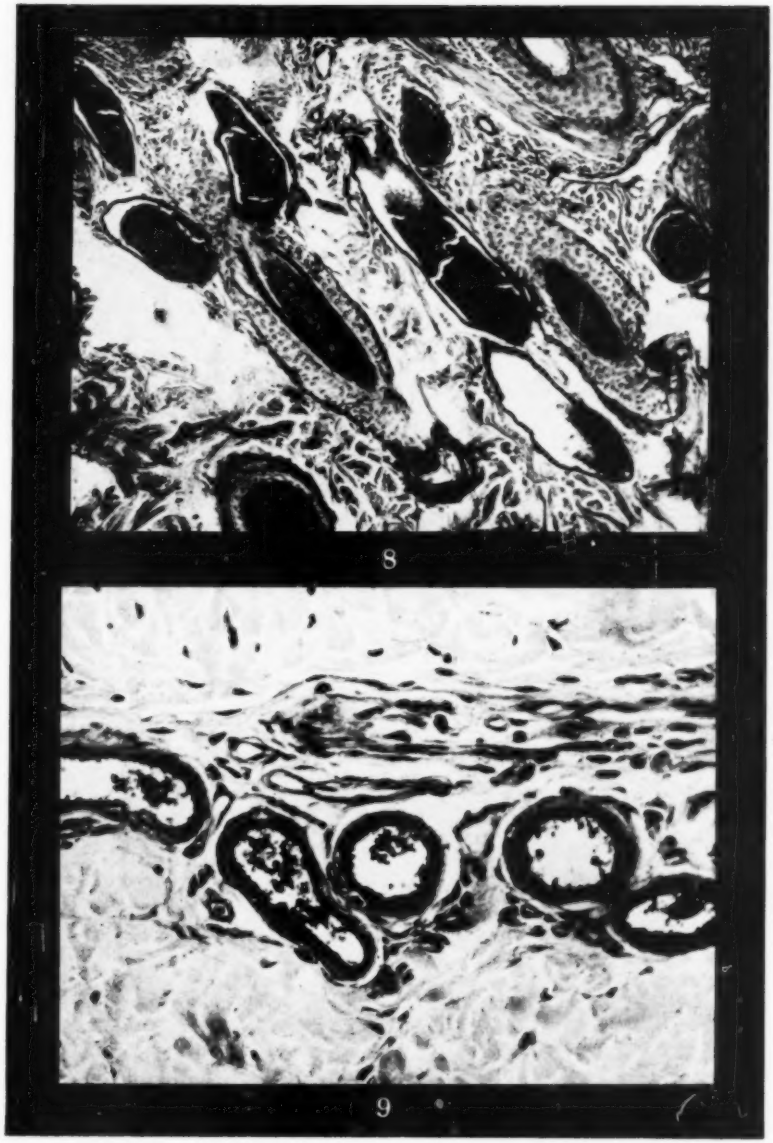

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Lennox, Pearce and Symmers.

THE EFFECT OF ADRENOCORTICOTROPHIC HORMONE (ACTH), CORTISONE AND HYDRO-CORTISONE ON THE GROWTH OF EXPERIMENTAL LYMPHOID TUMOURS IN CHICKS.

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A LIVELY interest in the relationship existing between lymphocytic leukaemia and lymphoid tumours on the one hand and the adrenal glands on the other, followed the observation that involution of normal lymphoid tissue and lymphopenia can be achieved by the administration of adrenocorticotrophic hormone (Dougherty and White, 1943) and adrenal steroids (Dougherty and White, 1944). A considerable mass of literature on the subject has accumulated. This has been reviewed elsewhere with regard to experimental lymphoid tumour (Donald and Higgins, 1951), human leukaemia (Adams, Valentine, Barratt and Lawrence, 1952) and normal lymphoid tissue (Yoffey, 1950). In humans the most encouraging effects of treatment with ACTH and adrenal steroids seem to have been temporary remissions, while some cases proved completely refractory. In experimental mammals the results likewise have varied between marked retardation of tumour growth during at least some period of treatment, decrease of tumour incidence after experimental transfer, and somewhat increased survival time, and, on the other hand, no notable change according to these criteria.

In chicks the effect of cortisone on the experimental lymphoid tumour Strain RPL 12 was investigated (Twelfth Annual Report of the Regional Poultry Research Laboratory, 1951). A dose of 5 mg. was given daily in one group from the time of tumour appearance, and in another group beginning at tumour inoculation. No retarding effect on tumour-growth was observed but the treated birds died earlier than the controls, apparently as a result of bacterial infection.

In this report the influence of ACTH, cortisone and hydrocortisone, on the growth of the lymphoid tumour Strain RPL 19, will be described. This strain was developed from a case of spontaneous visceral lymphoid tumour (Burmester and Denington, 1947 ; Burmester, 1947).

MATERIAL.

Crossbred Brown Leghorn × Plymouth Rock chicks, of both sexes, of 2 to 3 days of age were used. The sexes were randomised. The body weight of the chicks at the beginning of the experiments was approximately 40 g.

The hormone preparations used were adrenocorticotrophic hormone (ACTH RMC) manufactured by Roskilde Medical Company, cortisone acetate (also known as 11-dehydro-17-hydroxycorticosterone, Kendall's Compound E, " Cortone ") and hydrocortisone acetate (known alternatively as 17-hydroxycorticosterone,

Kendall's Compound F, " Hydrocortone "). The two last-mentioned hormones were manufactured by Merck & Co.

METHODS.

It was considered to be of advantage if the tumour volume could be used as a criterion of therapeutic effect, and that this volume could be estimated in living chicks, making daily observations possible. This necessitated a tumour site where most of the tumour area was available for inspection and measurement. Such a site is the wing fold in front of the elbow-joint. Here, on an area of about one square centimetre in the new-born chick, skin virtually lies upon skin, giving a very thin (about 1 mm. thick) fold, where, with light falling through, details like small vessels and the just developing tumour can be examined. After the feathers had been removed from this area, about 0·05 ml. of a tumour suspension, containing ground liver tumour and physiological saline in proportions 1:10, was injected near the fold edge. An equal number of control chicks were treated in a corresponding way, but here the suspension was made from normal liver tissue.

The ACTH dissolved in sterile water, was injected by a subcutaneous-intramuscular route. So as to avoid large volumes of solution, a micrometer syringe (" Agla ", made by Burroughs, Wellcome & Co.) was used. The volumes injected each time were in the order of 0·01 ml. The controls were given the corresponding volume of physiological saline.

In order to avoid the inhibitory effect on body growth of adrenal hormones, cortisone and hydrocortisone were applied locally. The quantity necessary to cover one side of the wing fold was found to be about 0·005 ml. This volume, corresponding to 0·125 mg. cortisone or hydrocortisone, was placed on each side of the fold and slightly massaged into the skin by means of a glass rod. The controls were treated in a corresponding way with the base solution in which cortisone is otherwise suspended.

Each experiment consisted principally of 4 different groups of chicks, namely, (1) tumour-inoculated and hormone treated, (2) tumour-inoculated and treated with blank, (3) inoculated with suspension of normal liver and hormone treated, and (4) inoculated with suspension of normal liver and treated with blank.

ACTH was applied four times, cortisone and hydrocortisone twice daily. In all experiments the treatment was initiated at the time of inoculation of tumour and carried out during the whole experiment.

Resulting from the similar resistance exerted by the skin of the wing-fold from above and below the developing tumours are usually ellipsoidal in form. The tumour body can thus be looked upon as composed of two sphere segments. Its thickness ($= 2\,h$), i.e., the height of the two segments, and diameter were determined with a calliper. Often the form of the segments, seen from above, was not circular but oval. Here the average of the two diameters ($= 2\,z$), perpendicular to each other, was taken. The volume of the tumour was hereafter estimated according to a formula, volume

$$= \frac{\pi h^2}{3}\left(h + \frac{3z^2}{h}\right)$$

which was derived from the formula for calculating the volume of the segment of a sphere.

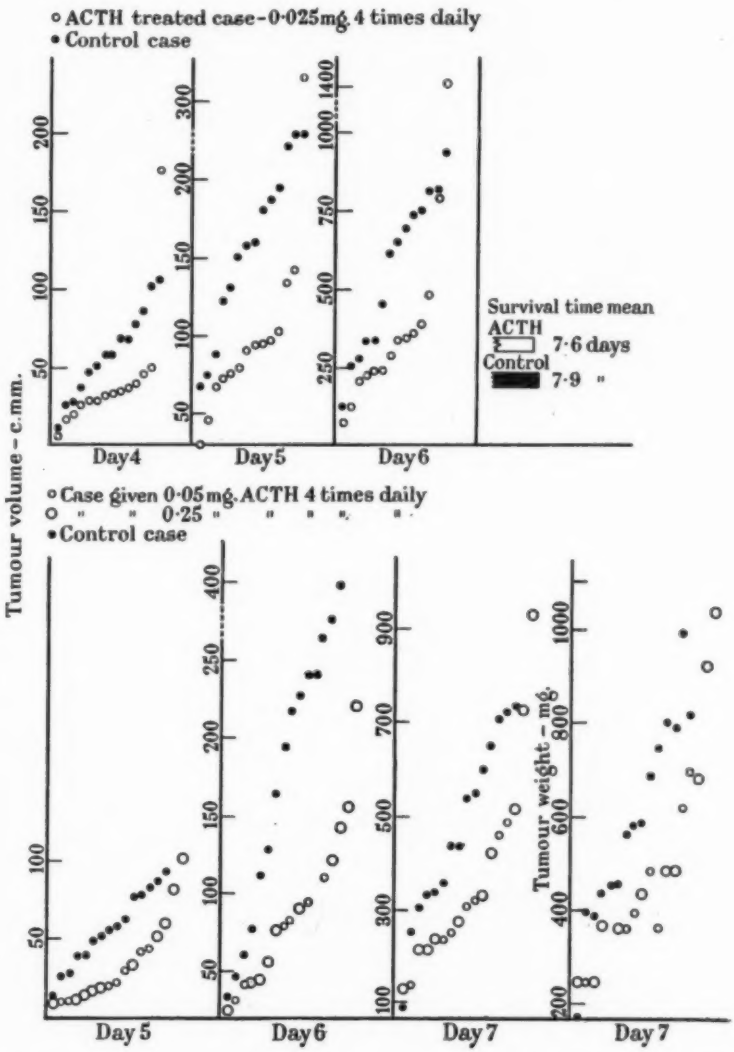

FIG. 1.—Influence on tumour volume of ACTH treatment. Day of tumour inoculation = day 0. Number of hormone-treated tumour-bearing chicks in the first experiment (upper section) was 14, number of tumour-bearing controls 14. In the second experiment (lower section) the numbers were 17 and 15 respectively. In the fourth column of the lower section the individual cases are arranged in the same order as in the third column to show the relation between calculated volumes and weights.

The mean volumes (c.mm.) are (ACTH-treated groups are mentioned first) for the upper section: (day 4) 40·9–58·6, (day 5) 103·1–156·7, (day 6) 431·0–555·1, and for the lower section (day 5) 35·0–57·1, (day 6) 89·5–179·0, (day 7) 365·4–471·3.

RESULTS.

Treatment with ACTH.

The results of two experiments with ACTH are shown in Fig. 1. The calculated tumour volumes are arranged according to increasing size. The general pattern of

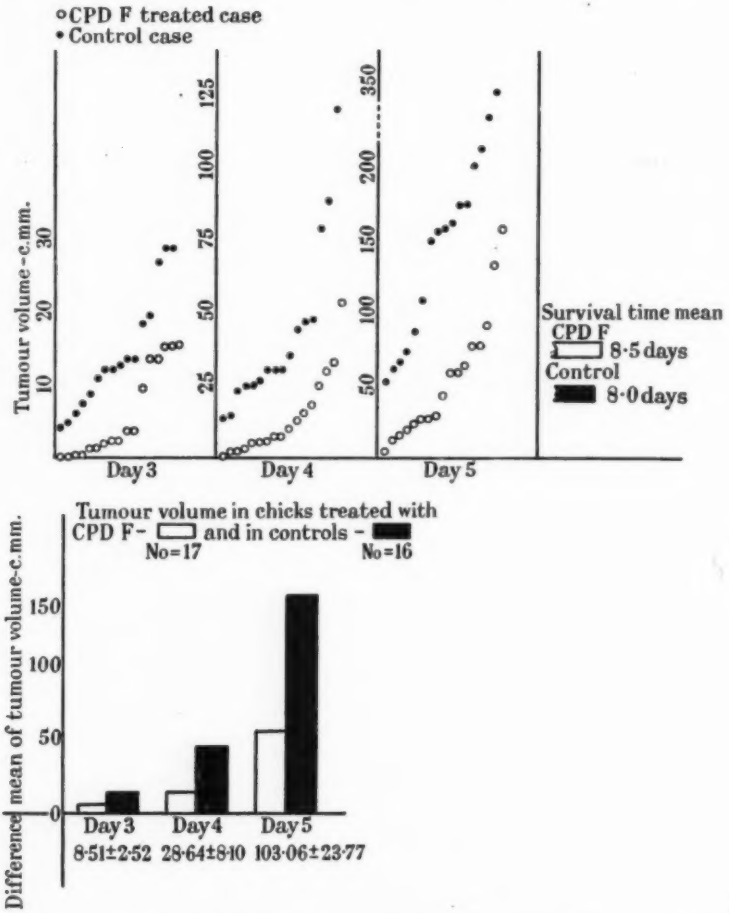

FIG. 2.—Influence on tumour volume of hydrocortisone (Compound F), treatment locally. In the upper section the cases are arranged as in Fig. 1 according to increasing size and for three consecutive days. From the mean values (lower section) it can be seen that in both groups the daily increase in volume was about three times that of the preceding day.

suppression of tumour size is preserved throughout the two experiments. In the first (upper section) 0·1 mg. ACTH was given daily. When in the second experiment (lower seotion) this dose was increased twice (to 7 chicks) or ten times (to 10

chicks) this did not result in a more marked inhibition of tumour growth. In no case did we notice any significant difference in time of appearance of tumours.

The survival time was determined in the first experiment. As the figure (upper section) indicates, the ACTH-treated chicks lived slightly, though not significantly, shorter than the controls.

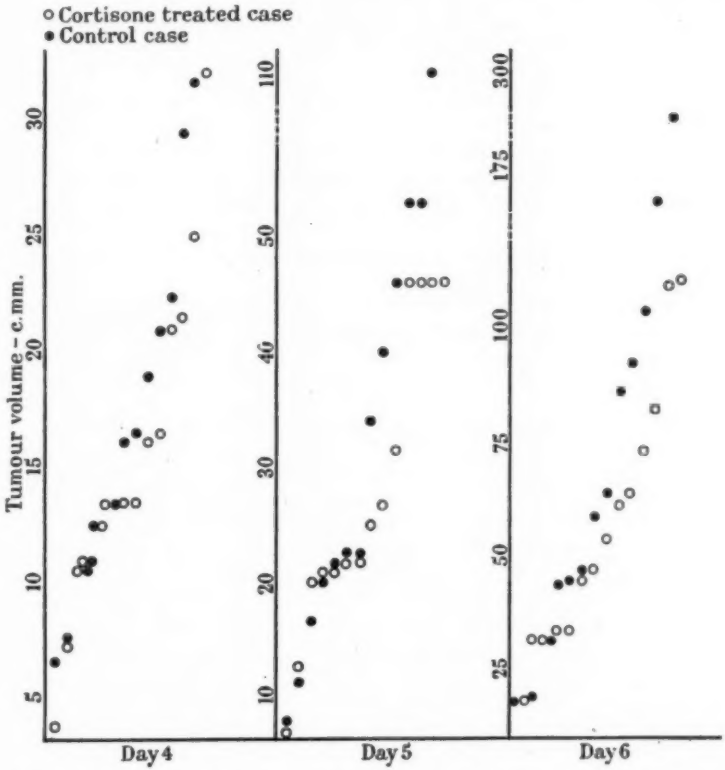

FIG. 3.—Influence on tumour volume of cortisone treatment locally. The cases are arranged according to the same principle as in the preceding figures. The mean volumes are (the cortisone-treated group is mentioned first) : (day 4) 16·3–16·8, (day 5) 29·5–35·3 and (day 6) 59·0–82·0.

In the second ACTH-experiment all chicks were killed on day 7 immediately after measuring of the tumours. These were dissected free and weighed on a torsion balance. The weights are given in Column 4 of the lower section of Fig. 1, with the cases arranged in the same order as in Column 3. Though a few weights deviate markedly from the " regression line " thus formed, it is evident that the agreement between the calculated volumes and the weights is good.

In both ACTH experiments the increase in body weight of the hormone-treated chicks was slightly less than that of chicks treated with physiological saline.

Treatment with hydrocortisone.

The results of local application of hydrocortisone are shown in Fig. 2. Tumours appeared in the control groups after (mean value) 3·00 days, in the treated group after 3·53 days. The difference, $0{\cdot}530 \pm 0{\cdot}173$ days, is statistically significant ($P < 0{\cdot}01$).

The difference in survival time (upper section) is not significant. The differences in tumour volume between the 2 groups for the 3 consecutive days (lower section of Fig. 2) are all statistically significant ($P < 0{\cdot}01$, $< 0{\cdot}01$ and $< 0{\cdot}001$ respectively).

Treatment with cortisone.

The effect on tumour volume of local cortisone treatment is shown in Fig. 3. The number of hormone-treated tumour-bearing chicks was 14 and of tumour-bearing controls 13. The very slight inhibitory effect of cortisone, shown in the figure, is not statistically significant.

No difference in time of first appearance of tumour or in survival time was observed.

Neither the hydrocortisone- nor the cortisone-treated chicks showed retarded body growth in comparison with their controls.

DISCUSSION.

It is evident from the figures that ACTH and hydrocortisone exerted a marked retardation on tumour growth, whereas the cortisone effect was very slight. This is in accordance with earlier observations (Karnofsky, 1950) that hydrocortisone is much more effective than cortisone in retarding body growth of young chicks The similar effect of ACTH and hydrocortisone in our experiments gives further support to the supposition that hydrocortisone and not cortisone is the hormone normally produced by the adrenals.

From Fig. 1 it can be concluded that an increase of the dose from about 2 mg. to about 20 mg. ACTH per kg. body weight did not result in a more marked retardation of tumour growth. The limiting factor here might be the maximum ability of the adrenal glands to secrete steroids. That this is not the case, however, is indicated by the following result of treatment with very large doses of cortisone. In one experiment, arranged as previously described, 10 mg. of cortisone per chick was injected (body weight at the beginning of the experiment was about 75 g.). After one week the chicks were killed and body weights and tumour weights were determined. The blank treated animals had increased their body weight by about 50 per cent of the initial weight. The cortisone chicks had not increased but slightly decreased their body weight. The mean tumour weight, however, was 3·88 g. in the control group and in the cortisone-treated group 3·23 g. Thus even a dose of cortisone, which completely retarded body growth, only inhibited tumour growth very slightly.

This brings us to the question of the mechanism of the steroid inhibiting action of which little is known. Cortisone strongly inhibits mitotic rate of normal epidermis, and it is suggested that this is a result of interference with carbohydrate metabolism (Bullough, 1952).

The experiment just described shows that cortisone may almost completely fail to inhibit growth in a host, whose own body growth is definitely arrested. It

is well known that glycolysis plays a greater role in the carbohydrate downbreak in tumours and embryos than in normal adult tissue, and there are strong indications that while mitosis is totally inhibited in adult normal tissue in the absence of respiration, it can proceed in tumour and embryonic tissue under such conditions (Bullough, 1952). The inference from this is that if the adrenal steroids exert their inhibiting effect on carbohydrate utilisation in the aerobic phase, their above-described effect on tumour growth could be explained. This would afford an explanation of the interesting observation by Karnofsky (1950) that chick embryos under 8 days of age did not respond to cortisone with growth inhibition while embryos above that age did. Cells of young embryos and tumour cells can apparently switch over to a metabolic pathway in cell division which is uninfluenced by adrenal steroids, whereas cells of older embryos and normal adult cells cannot.

The following working hypothesis would thus be established. The adrenal steroids inhibit Kreb's cycle at some point. Only cells which can utilise glycolysis for division can go on dividing. Though this hypothesis may seem to be based on weak premises, it would fit in well with the very temporary inhibition which the hormones produced in our experiments. This can be especially well studied in Fig. 2. The differences in tumour volumes are significant for each day measured, but it is evident that the inhibition had already occurred before and did not go on while the measurements were performed. The lower section of the figure shows that the tumours of both groups grow during each day to about three times their size on the preceding day. The same is the case, though not so pronounced, in the ACTH and cortisone experiments.

In a report on the effect of adrenalectomy and cortisone on the intermediates of the Kreb's cycle (Umbreit, 1951) there is, however, nothing which supports our hypothesis, except that interference of the hormone really seems to occur in this stage of carbohydrate metabolism.

SUMMARY.

The effect of ACTH, cortisone and hydrocortisone on experimental lymphoid tumour (RPL 19) in chicks was examined. The tumour material was injected into the wing fold, where subsequent measurements on living chicks could conveniently be performed. The steroids were applied locally in order to avoid interference with body growth. ACTH and hydrocortisone exerted a marked but temporary inhibition on tumour growth. Cortisone affected the tumour growth only very slightly.

The possible mechanism of action by interference of the adrenal steroids with the carbohydrate metabolism is discussed.

We are grateful to Merck & Co., Inc., and to the Medical Research Council for supplying the hydrocortisone and cortisone used in these experiments.

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AMETHOPTERIN: A TOXIC TUMOUR GROWTH INHIBITOR.

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THE introduction of the toxic analogues of pteroylglutamic acid in the treatment of human leukaemias reported first by Farber, Diamond, Mercer, Sylvester and Wolff (1948), and subsequently by many other workers, has led to an extensive investigation of the properties of these compounds. The rather scanty information which was at first available to afford theoretical support for the rationale of this treatment has become expanded into a literature of considerable proportion. Burchenal and his associates (1951) and Petering (1952) have reviewed the more important investigations into the properties of pteroylglutamic acid analogues and quote references to the work which has substantially reduced the empiricism of this chemotherapeutic approach. Although much enthusiasm has been developed for the antineoplastic potentialities of these compounds, their usefulness in treatment has been greatly limited by their extreme toxicity, perhaps the most striking of their properties. Their application is being increasingly restricted on this account.

The very poisonous character of these drugs has received much detailed consideration in the clinical context, but in common with other toxic drugs used in this field, less theoretical attention has been directed towards the problems connected with the assessment of their usefulness than would seem to be justified in view of the importance of this aspect of chemotherapy. In the great bulk of experimental work, 4-amino-N_{10} methyl pteroylglutamic acid (amethopterin) and 4-amino pteroylglutamic acid (aminopterin) have been used. The former, being some ten times less toxic than aminopterin (Ferguson, Thiersch and Philips, 1950), has been used in the work reported here.

Sugiura, Moore and Stock (1949) reported that aminopterin produced growth retardation of a transplanted sarcoma, an adenocarcinoma and a melanoma in mice, and noted that inhibition only occurred at toxic levels of dosage. It was suggested, nevertheless, that this association between the tumour retarding effect and the toxicity was compatible with the view that aminopterin had a definite effect against tumour tissue. Schoenbach, Goldin, Goldberg and Ortega (1949) have also reported growth regressions of Sarcoma 180 in response to aminopterin and observed that body weight losses of up to 20 per cent were sustained by the mice, but concluded from the greater percentage loss sustained by the tumours that the drug was acting selectively. Moore, Stock, Sugiura and Rhoads (1949) obtained inhibition of the growth of Sarcoma 180 using amethopterin and noted the association between toxicity and tumour growth retardation. The importance of this was not believed to be great enough to modify the conclusion that the compound was a powerful inhibitor of the growth of this tumour. The evidence presented here, though necessarily circumstantial, supports the contention that most of the anti-neoplastic activity of amethopterin for Sarcoma 180 and Sarcoma

37 is the result of an unselective toxic state induced as a result of treatment in the animals carrying the tumours. The sex difference in toxicity reported by Goldin, Greenspan, Goldberg and Schoenbach (1950) was confirmed and it was noted in preliminary experiments that tumour growth retardation was obtained only in tumours carried by the male mice which also showed a lower tolerance to the drug. It was decided to investigate this sex difference in order to discover whether the difference in the growth of the tumours between the sexes was a function of the sex of the host acting by enhancing the effect of the drug on the tumour or whether it was due to a secondary effect caused only by the different susceptibility to intoxication.

MATERIALS AND METHODS.

The mice used throughout were mixed strain albinos obtained from reliable dealers and used when they had attained a body weight of between 25 and 30 g. They were housed in an air-conditioned atmosphere, the temperature of which was maintained at 24 $\pm$ 4° C. Wire cages of a size comfortably to accommodate 20 mice were fitted with wire floors carrying bent wire feet so that the cages were raised about 2 cm. above the level of the metal trays in which they were placed. Each tray was lined with a sheet of absorbent paper. Water was supplied *ad lib.* from bottles fitted with long stems which reached to within 2 cm. of the floors of the cages. The rat cake used had the following composition :

Moisture	14·3 per cent.
Soluble carbohydrate	53·4 ,,
Protein	20·0 ,,
Fat	3·8 ,,
Fibre	3·3 ,,
Ash	5·2 ,,

It was finely ground in a mill and dried at 37° C. for 24 hours in a thin layer. The degree of desiccation obtained in this way was found to be such that there was a negligible alteration in the water content of the ground food when this was exposed to the conditions existing in a cage of mice for 24 hours. A weighed excess of the food was given to the mice in a suitable arrangement of 2 oz. and 4 oz. glass ointment jars fitted with metal screw caps in each of which a circular hole, 3 cm. in diameter, had been cut to allow access to the food. On the surface of the food was placed a wire grille of such a size that it easily fell in the jar as the food was eaten, but could not be pulled through the hole in the screw cap. This arrangement made it easy for the mice to reach and eat the food, but difficult for them to scatter more than a small amount. Contamination of the food in the jars was rarely seen and was always trivial in extent. Any food which was scattered was collected on the paper on which the cage rested. The jars were filled each day at the same time and the food remaining in them was weighed. The papers contaminated with urine, faeces and scattered food, were collected and dried in the air for 4 days, after which time the scattered food was separated from the faeces by rubbing the mixture through a 12-mesh sieve. Though tedious and rather distasteful, no real difficulty was encountered in making a complete separation. The food consumption was then obtained by difference. All the weighings of food were recorded to the nearest 0·25 g.

The mice were grouped according to weight so that in any one cage the weights varied from one another by no more than 3 g. The animals were weighed individually to the nearest 0·25 g. every other day throughout the experiment. They were allowed to become accustomed to life in a cage without shavings for one week before the experiment was started, when weighing of the food and the mice was begun and continued until the food intake was found to have become stabilized. At this point a selection was made in order to reduce the scatter among the body weights of the individuals in a cage. The extremes of weight were rejected and of the remaining animals exchanges were made between the cages if this would increase the uniformity. Implantation was done 2 days later.

Two sarcomas were used, Sarcoma 37 and Sarcoma 180. Sarcoma 37 was originally obtained from the Chester Beatty Research Institute of the Royal Cancer Hospital, and has been perpetuated in this laboratory for several years by serial implantation. Sarcoma 180 was obtained from the Sloan-Kettering Institute of New York through the courtesy of Dr. C. Chester Stock. This tumour was descended from the original Crocker Sarcoma 180. It was obtained from the United States after a series of ambiguous experiments with Sarcoma 37 had raised the possibility that this latter tumour might be resistant to the action of amethopterin.

Implantation of the Sarcoma 180 was done by the trocar-cannula method. Sarcoma 37 was implanted aseptically from a micrometer syringe by the method described elsewhere (Bennette, 1952). In every case 0·04 ml. of tumour mince suspension in 0·9 per cent NaCl was injected from a No. 14 needle subcutaneously into the right flank. In any one experiment all the mice were implanted with the same homogeneous tumour mince prepared by the method of Craigie (1949*a*) in a pressure mincer fitted with two plungers with groove depths of approximately 0·5 and 0·125 mm. In Experiments 1–4, a 50 per cent (v/v) mince suspension in saline was used.

Treatment was started 6 days after implantation in Experiments 1 and 2, and 10 days after implantation in Experiments 3 and 4. The sample of amethopterin used was obtained from Lederle Laboratories through the courtesy of Dr. T. H. Jukes. It was of tested purity. In all the experiments reported, solutions containing 12 mg. per cent (w/v) of amethopterin were made up freshly each day in sterile 0·1M NaH_2PO_4 : Na_2HPO_4 buffer at pH 7·8 and were stored at 0° C. Control animals received the same phosphate buffer alone. The volumes of the doses given, representing about 0·3 per cent of the body weight of the mice, were varied according to the mean weights of the groups so that the dose per kg. should be as nearly as possible the same for two groups of different mean body weight. At a site removed from the place of implantation, subcutaneous injections were given at 8-hourly intervals in accordance with the findings of the toxicity experiments.

At the end of the experiments surviving mice were killed and the tumours dissected out and examined for areas of necrosis, the degree of adherence to surrounding structures, the presence of ulceration and the vascularity. The growths were weighed wet to the nearest 10 mg. Systematic histological examination was not done in this series of experiments.

In Experiment 5, the procedure was identical to that described above except in the following respects. At the time of implantation, half the number of mice in each cage were distinguished from the rest so as to provide a total of 16 groups.

Control and experimental sets for each sex were implanted with the standard volume of tumour mince suspensions at the following dilutions (v/v) : $10^{-1.0}$: $10^{-1.5}$ $10^{-2.0}$: $10^{-2.5}$. Treatment was started 2 days after implantation. The appearance and development of progressively growing tumours were recorded for 19 days after implantation, during which time food intake and body weight records were made. When the mean body weights of the experimental groups showed that recovery from the effects of treatment was well established, food intake and body weight records were discontinued and the mice bearing tumours were killed. Those mice with no palpable tumours were kept under observation for another 23 days, after which it was assumed that no further tumours would appear and the animals were killed. The tumours were not weighed or subjected to post-mortem examination since it was the appearance of the growths and not the character of their subsequent development which was important in this experiment. The mice which died as a result of treatment were examined for the presence of tumours. Those which bore tumours were included in the analysis while those which showed no sign of a tumour were excluded.

Toxicity.

Details of the toxicity experiments are not presented. The findings of Ferguson, Thiersch and Philips (1950) were repeated. It was found that the tolerance was markedly reduced when the total dose was progressively fractionated. Thus, when the drug was given daily, the total dose tolerated was more than double that producing an equivalent condition of intoxication when the injections were given 8-hourly. The individual variation in the response of albino mice was considerable when the drug was given once daily, even in groups carefully selected for weight uniformity. This variation was substantially reduced by giving the injections three times a day. Reports of the rapid excretion of pteroylglutamic acid analogues quoted by Burchenal *et al.* (1951) and the findings of Swenseid, Swanson, Miller and Bethell (1950) support the view that the maintenance of an effective blood level would be favoured by reducing the intervals between injections. The subcutaneous route was chosen for the same reason.

CALCULATION AND INTERPRETATION OF RESULTS.

The mice comprising some of the control and all of the experimental groups were housed in more than one cage, and since it was necessary to make selections in order to reduce the scatter among the individual body weights of the animals, it was not possible to regard the groups as constituting a single population. The body weight curves were accordingly calculated for each cage separately and then t-tested one against the other. In most cases no significant difference was found and grouping was therefore possible. In the following sets however—Experiment 2 female experimental : Experiment 4 male experimental : Experiment 5, male control—small but significant differences were found between the body weight curves derived from different cages. In each case the displaced curves pursued courses roughly parallel to the others up to the time of treatment, and it was considered likely that a simple procedure for making a correction for the displacement could be justified. The mean difference between corresponding points on the curves was obtained and the assumption that this value represented the extent of a simple displacement of the origin was tested by repeating the t-test

on the pairs of points obtained by appropriately adding or subtracting the mean difference. No significant difference was found in any of the instances after the correction had been made and it was considered unnecessary to do a full analysis of covariance. Having obtained single curves for the grouped control and grouped experimental sets for each experiment, these were now tested for significant difference up to the onset of treatment. In Experiment 3 (females) and Experiment 5 (males and females) significant displacements were found to exist. These were corrected for as has been described, the mean difference being added in each case to the curve with the lower origin. As before the procedure was justified by the finding that no significant differences existed after the corrections had been made. The estimated terminal mean body weight values were corrected for the presence of the tumours by subtracting the estimated mean tumour weights from the terminal mean body weight values, and the body weight changes were expressed as percentage reductions on the mean control values. Since the control tumours never reached such a size as to cause any obvious constitutional disturbance in the mice, it could be assumed fairly confidently that their presence had a comparatively small effect on the body weight. The error due to the difference in tumour sizes between control and experimental animals, if their presence were responsible for a loss in body weight, would result in an understatement in the percentage weight change obtained. The temporary depression or flattening of the ascending weight curves of the control groups, corresponding to the period of injections, may be taken to be due to the disturbance produced by the extra handling involved. The body weight figures refer only to those mice which survived the experiments, including those described as moribund at the end. This has been done in order to exclude the " effect of death " on the body weight, a sudden profound terminal loss due to dehydration. In a few cases the moribund mice may have been in this state, but the easy access to water would mean that mice in an advanced toxic state would have to be virtually immobilized before terminal dehydration could set in. Mice referred to as " moribund " were those within, say, 48 hours of death—ruffled, unsteady and suffering in most cases from diarrhoea which was haemorrhagic in some instances. A distinction is drawn between the use of the words " moribund " and " dying." It was found in most cases that a ruffled, unsteady mouse described as moribund would pass into an agonal state in which it would become grossly ataxic and finally cease to be able to move about the cage. The rate at which this sequence of events occurred was rather variable, and in order to account for this, an arbitrary correction was made by excluding dying mice from the numbers of the groups on the day before their death when calculating the food intake per mouse for that day. The food intake figures were obtained from data relating to the whole group. The mean food intake per mouse was calculated for each cage separately over 4-day periods, and the results were subjected to the same analysis as that applied to the body weight results. The procedure proved to be satisfactory in every case but one (Experiment 3, females). In this group there were significant differences before, during and after treatment, and these were not consistent. The result has been recorded as of doubtful significance.

The quantitative estimates set down in Table I were derived from data for which the values of the significance P were in every case less than 0·01. A less exacting criterion of significance is regarded as being inappropriate in work of this kind.

TABLE I.—*Showing Relation Between Toxicity and Tumour Inhibition Due to Amethopterin.*

Experiment No. and description of group.			Number of injections.	Total dose mg. per kilo.	Body wt. loss $\frac{\text{con.-exp.}}{\text{con.}} \times 100$.	Food intake reduction $\frac{\text{con.-exp.}}{\text{con.}} \times 100$.	Number of deaths.	Number terminally moribund.	Tumour wt. loss $\frac{\text{con.-exp.}}{\text{con.}} \times 100$.	Total take rate.
1 S180	♀	Con.	10	—	(0·05 < P < 0·1)	12·9 ± 2·6	0/14	0	(0·02 < P < 0·05)	88/90
		Exp.		4·0			1/30	0		
	♂	Con.	10	—	13·3 ± 3·4	42·4 ± 5·5	0/15	0	. 38·0 ± 10·0	
		Exp.		4·0			5/29	12		
2 S 180	♀	Con.	13	—	17·5 ± 2·0	45·2 ± 5·3	0/15	0	. 58·5 ± 8·5	85/90
		Exp.		5·2			7/27	9		
	♂	Con.	8	—	(0·1 ± P ± 0·2)	(0·1 < P < 0·2)	0/15	0	(0·2 < P < 0·3)	
		Exp.		3·2			2/28	0		
3 S 37	♀	Con.	10	—	(0·1 < P < 0·2)	(Doubtful significance)	0/11	0	(0·2 < P < 0·3)	103/105
		Exp.		4·0			0/20	0		
	♂	Con.	10	—	13·6 ± 1·7	34·2 ± 5·2	0/10	0	. 38·5 ± 8·0	
		Exp.		4·0			2/22	14		
4 S37	♀	Con.	13	—	16·9 ± 1·8	54·8 ± 5·6	0/10	0	. 57·1 ± 9·5	119/120
		Exp.		5·2			11/33	13		
	♂	Con.	8	—	(0·4 < P < 0·5)	9·8 ± 3·1	0/9	0	(0·05 < P < 0·1)	
		Exp.		3·2			1/29	0		
5 S37	♀	Con.	13	—	23·5 ± 1·3	61·8 ± 4·2	0/37	TPD_{50} calculated from number of takes at mince dilutions $10^{-1\cdot0}$; $10^{-1\cdot5}$; $10^{-2\cdot0}$; $10^{-2\cdot5}$	$10^{-1\cdot95 \pm 0\cdot22}$	
		Exp.		5·2			13/37		$10^{-1\cdot66 \pm 0\cdot23}$	
	♂	Con.	9	—	11·2 ± 2·3	52·4 ± 4·1	0/36		$10^{-1\cdot89 \pm 0\cdot23}$	
		Exp.		3·6			8/40		$10^{-1\cdot79 \pm 0\cdot24}$	

$\frac{\text{Con.-Exp.}}{\text{Con.}} \times 100$ signifies the ratio of the difference between the estimated mean values for the control and experimental groups to the estimated mean control value, expressed as a percentage. Quantitative estimates have been given under this heading only in cases where the control and experimental values differed significantly, taking $P = 0{\cdot}01$ as the criterion of significance.

TPD_{50} represents the estimated dilution of tumour mince which would have produced tumours in 50 per cent of the mice.

Fig. 1 and 2 show the food intake and body weight curves in Experiments 3 and 2 respectively. The corresponding curves for Experiments 1 and 4 are essentially the same. Since all the corresponding points have been t-tested, the

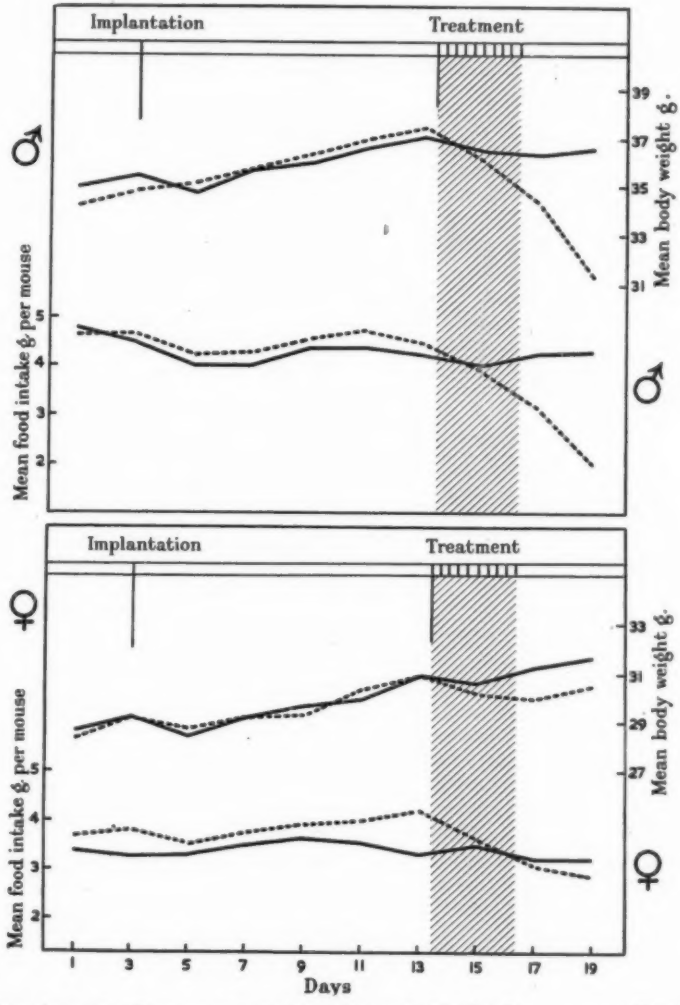

FIG. 1.—Experiment 3. Sarcoma 37. Body weight and food intake. - - - - - - Treated group. ——— Control group.

individual values for the standard errors about the means have been omitted from the figures for the sake of clarity.

In Experiments 1 and 3, the males and females received exactly the same

treatment and it is seen that it was only in the males that any important degree of intoxication occurred and that tumour growth retardation likewise only occurred to a significant extent in these groups. In Experiments 2 and 4 the does

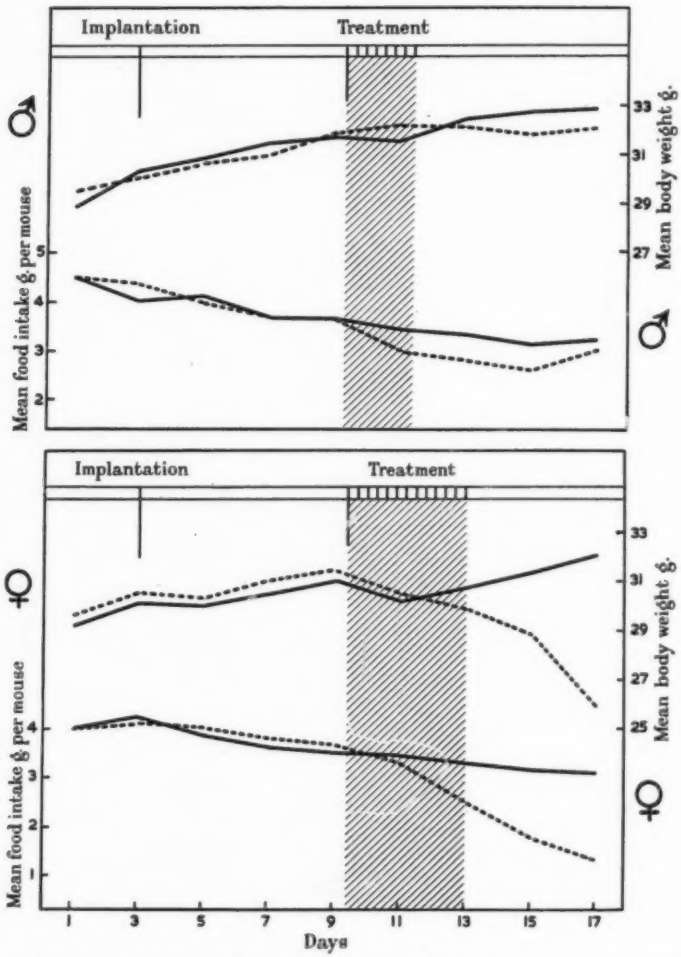

FIG. 2.—Experiment 2. Sarcoma 180. Body weight and food intake. ------ Treated group. ——— Control group.

given to the females was increased and that given to the males decreased, with the result that the females exhibited the manifestations of toxicity and also showed a retardation of the growth of the tumours they carried. The males showed no such changes to any important extent. For the males, a 20 per cent reduction in the total dose producing intoxication and tumour growth retardation

failed to produce either to a significant degree. For the females the corresponding figure was 23 per cent.

Micrococci and beta-haemolytic streptococci were found to have contaminated the minces used for implanting the tumours in Experiments 3, 4 and 5, in spite of aseptic precautions. This problem was encountered by Warner and Gostling (1950) and in common with these workers it is felt that while it is important to check the degree of contamination, the presence of bacteria does not necessarily invalidate the results when experiments are adequately controlled.

No obviously consistent differences were found between the naked eye appearances of control and experimental tumours and the records have not been analysed in detail.

In Experiment 5, the 50 per cent end point of the " titration ' has been calculated by Thompson's method (1947), this being recommended by Armitage and Allen (1950) as a reliable short-cut method in which the result can be expressed within 95 per cent fiducial limits. The index used by Warner and Gostling (1950) has not been calculated. Since the standard error of the difference between the "tumour producing dose $_{50}$" (TPD_{50}) for the control and experimental sets is greater than the difference itself, the shifts in the end points as a result of treatment at a toxic level of dosage cannot be regarded as significant.

DISCUSSION.

The results of Experiments 1 to 4 show that retardation of the tumours Sarcoma 180 and Sarcoma 37 only occurs to an important extent when the hosts are intoxicated by amethopterin to the point where about half the animals have either died or passed into an advanced toxic state as a result of treatment under the conditions stated. The question to be decided is the extent to which the action of the drug can be regarded as selective in its effect upon the tumours. Lees and Lees (1950) repeatedly found that non-specific toxic substances with low therapeutic ratios would bring about " significant " retardations of tumour growth. One main purpose of this report is to submit an additional emphasis to the recommendation made by these workers that any drug with a low therapeutic ratio may be responsible for unjustifiable optimism as to its tumour retarding activity if the basic considerations of the therapeutic ratio are not fully taken into account. Rous (1914), Bischoff, Long and Maxwell (1935), Bischoff and Long (1938), Tannenbaum (1947) and Goldin and his associates (1949) are among those who have shown that the growth of recent implants is markedly affected by restriction of the caloric intake. The problem of deciding between selective and unselective toxicity has been approached by Goldin and his co-workers (1949) in experiments with nitrogen mustards. These workers have studied the effect of dietary restriction on the growth of Sarcoma 180 and concluded from paired feeding experiments that the weight loss incurred by the treated animals could account for the whole of the tumour growth retardation observed in every case but one of a series of toxic nitrogen mustards. By reference to a regression line relating the percentage tumour weight and the percentage body weight loss, it was stated that only 29 per cent of the total tumour regression due to this one compound could be attributed to a specific effect of the drug.

The use of a regression line as a means of assessing the utility of a toxic chemotherapeutic agent may be useful as a means of excluding compounds the toxicity of which is such that the body weight loss caused by their use can be shown to be

sufficient to bring about the whole of the tumour growth retardation observed. But where a retardation occurs which is greater than can be accounted for in this way, the assertion that this residual activity is specific implies that body weight loss is taken to be a completely satisfactory criterion of chronic toxicity. Paired feeding experiments designed to establish regression lines for the tumours used in the work reported here were not done because it is held that no single criterion is adequate for the quantitative expression of chronic poisoning. Furthermore, it cannot be assumed that information derived from calorie restriction experiments can be applied directly to cases where the reduced food intake is the result of anorexia due to slow poisoning, with all the complex metabolic disorder which must occur in such a state. The use of a regression line as a means of making a quantitative correction for the effect of calorie restriction on the growth of a tumour is felt to be of little use if it remains impossible to have confidence that the residual activity really represents a specific action on the part of the drug. If the effects of intoxication on the growth of a tumour cannot be separated from a supposedly selective effect of the drug causing the intoxication, it must also be impossible to apply the conventional approach of chemotherapy to the study of animals bearing tumours—that is, the attempt to enhance the specific action and reduce the toxicity by correlating the biological activity with the chemical structure. On theoretical grounds, then, it is submitted that the study of the effect of drugs on the growth of tumours in animals is not profitable when the compounds are active only at lethal or almost lethal levels. Such a conclusion is by no means original, but no apology is offered for its restatement because it is clear that much careful work continues to be done without full recognition of the fact that the results in many instances cannot be interpreted unequivocally by reason of these considerations.

The titration method used by Warner and Gostling (1950) was adopted as a means of investigating the effect of amethopterin on the tumour producing potency of a mince of Sarcoma 37. Exposure of a dispersed implant to amethopterin at a toxic level shortly after implantation was thought likely to represent a condition in which the tumour cells would have the maximum susceptibility to damage. The fact that no significant alteration in the estimated 50 per cent end point was discovered is surprising in view of the findings of Tannenbaum (1947) relating to the effect of calorie restriction on the early development of tumours. But the failure by this method to demonstrate an action of the drug on the tumour cells is not surprising in view of the failures reported in attempts to show a specific action *in vitro* (Stock, Biesele, Burchenal, Karnofsky, Moore and Sugiura, 1950). The titration method has the advantage of overcoming many of the difficulties inherent in the quantitative evaluation of tumour growth in terms of growth rate. Its applicability to experimental cancer chemotherapy is being investigated. The criticism of the method advanced by Craigie (1949*b*) has been noted. It would appear that the objection applies more particularly to cases where comparisons are attempted between experiments in which difference minces have been prepared.

The negative result of Experiment 5 taken in conjunction with the fact that a reduction by one fifth of a toxic and tumour retarding dose produces neither tumour inhibition nor signs of toxicity under the conditions of the experiments is taken to indicate that the association between toxicity and tumour inhibition is too close to make it possible to place any reliance on the selectivity of the tumour retarding effect of amethopterin for the transplanted Sarcomas 37 and

180. The difference between the conclusion reached here and that given by other workers in this field, notably Stock and his associates (1950), seems likely to be due essentially to the fact that the use of an 8-hourly treatment procedure made it possible to work in the neighbourhood of the extreme limit of tolerance to the drug and to define more narrowly the conditions prevailing at the toxic and just subtoxic levels of dosage. The possibility cannot be overlooked that tumour tissue might be selectively damaged when exposed to higher concentrations of amethopterin intermittently while it is resistant to a more continuous exposure to lower concentrations, such as would be produced by more frequent injections.

SUMMARY.

Experiments have been described in which the tumour retarding action of 4-amino-N_{10} methyl pteroylglutamic acid (amethopterin) in mice has been related to the toxic state induced in the animals as a result of treatment. The degree of chronic intoxication has been judged by the food intake and body weight changes which occur. The conclusion has been reached that the anti-neoplastic activity of the drug is so closely associated with manifestations of toxicity that no confidence can be placed in the selectivity of its action against the two transplanted sarcomas studied. The sex difference in toxicity has been confirmed and the failure to obtain a significant retardation of tumour growth except at the toxic levels of dosage for the two sexes supports the conclusion that the retardation is, to a great extent at least, the result of an unselective poisoning. Some of the problems connected with the evaluation of toxic chemotherapeutic agents in this field have been discussed.

The support of the British Empire Cancer Campaign, which has borne the expense of this work, is gratefully acknowledged. It is a pleasure also to extend thanks to Professor F. Dickens and Professor E. C. Dodds for their help and encouragement, to Dr. C. T. Beer for help during part of the experimental work, and to Dr. P. Armitage of the Medical Research Council's Statistical Research Unit for his advice in connection with the statistical analysis.

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A STANDARD PROCEDURE FOR IMPLANTING TUMOUR CELL SUSPENSIONS.

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THE need to standardize the procedure for implanting tumours when quantitative work is being done is specially important in experiments involving the use of the titration method, by means of which the tumour-producing potency of a suspension of tumour cells is estimated (Schrek, 1935 ; Shear, 1936 ; Warner and Gostling, 1950). In work in which the growth rate of tumours is being studied it is also an advantage, at least theoretically, to base the information gained on the knowledge that the implantation was done by a uniform method. This is particularly so when tumours are implanted into pure strain animals in which their growth tends to be more uniform than in mixed strain animals.

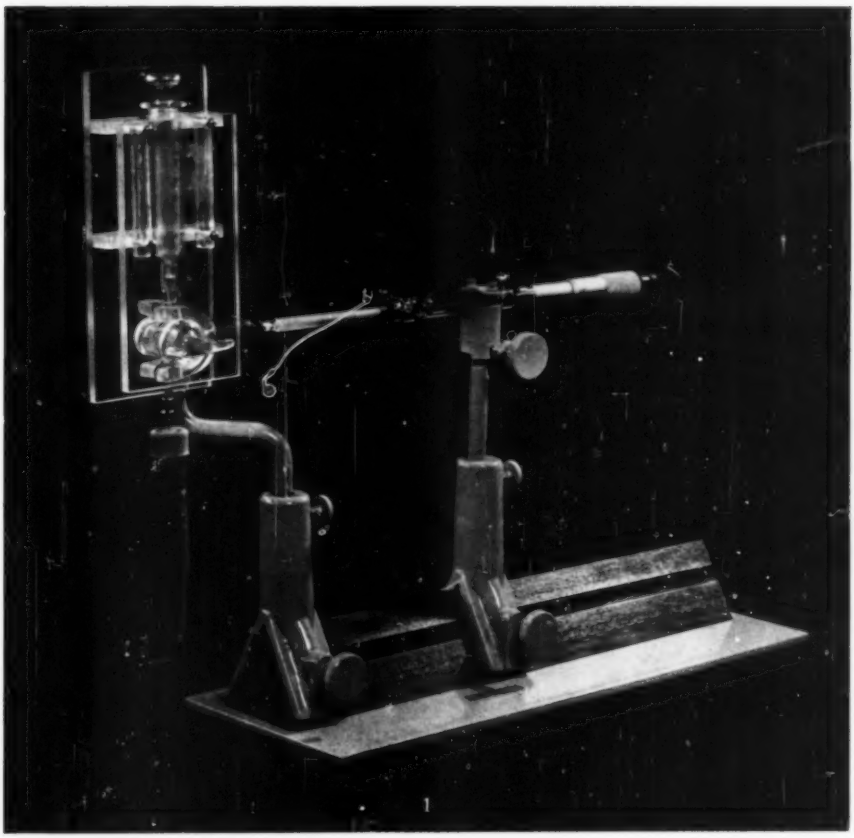
To this end, use has been made of the Agla micrometer syringe (Burroughs Wellcome) which is designed to deliver accurately uniform volumes. With the help of a device to facilitate aseptic manipulation of the tumour cell suspensions, a technique has been developed which makes it possible to implant tumours rapidly and accurately without the need of assistance.

The procedure used for routine implanting at one dilution will be described. A mince is prepared using Craigie's pressure mincer (Craigie, 1949). There is some evidence (Epstein, M. A., personal communication) that the best results are obtained when two plungers of grooves of depths 0·5 and 0·125 mm. are used. Pieces of tumour for mincing are taken from the peripheral parts of large but not ulcerated tumours. The operation is done aseptically and samples of the mince are plated on to blood agar before and after the implantation. A volume of mince measured as accurately as possible from a syringe, is transferred to a sterile rubber capped graduated centrifuge tube and sterile 0·9 per cent (w/v) NaCl is added to make a 50 per cent (v/v) suspension, using the graduations of the tube. The suspension is then centrifuged at 1000 r.p.m. for 2 minutes to remove minute air bubbles which remain in the suspension even after the addition of the diluent.

The micrometer syringe is mounted horizontally and rigidly on a stand. The mounting can be swivelled through 90°, the movement being controlled by a stop and a tightening screw. The all glass syringe supplied with the instrument has not been used. It is rather fragile for use in this context and the nozzle has so fine a bore that blocking by fibrous material in the suspension proved to be troublesome. Furthermore, the high degree of accuracy of which it is capable seemed inappropriate for this kind of work. An ordinary good quality Luer tuberculin syringe has been calibrated and has been shown to be well suited for attachment to the micrometer fitting. It was found to be more satisfactory to

fill this with the rather viscous suspension by driving this in rather than sucking it up by withdrawing the plunger in the usual way. The device used to transfer the mince suspension to the implanting syringe is shown in Fig. 1. A 3-way tap (1 mm. bore), fitted with the Luer ground joints on two of the side arms has been mounted rigidly between two clear Perspex plates. An all glass Luer 5 ml. syringe, referred to as the mixing syringe, fits into the socket joint above and is held firmly by the barrel. A 4 inch Luer exploring needle projects downwards from the cone joint below and passes through the rubber cap to the bottom of the tube containing the cell suspension. This tube is jacketed by a Dewar flask containing ice and water. To the third arm of the tap is attached a Luer fitting adaptor which is held firmly against the end of the side arm by a short piece of rubber tube which allows just enough flexibility to eliminate the risk of snapping off the glass tip of the micrometer syringe when this is inserted into the adaptor for filling. This part of the apparatus is mounted in such a way that the side arm adaptor can be adjusted to come into alignment with the micrometer syringe when this is rotated on its mounting. To fill the implanting syringe at the outset, it is necessary to take special care to exclude air bubbles. Thereafter the problem does not arise. The photograph shows the micrometer syringe in the filling position. The procedure for filling is as follows: the cell suspension in the reservoir is thoroughly mixed by drawing it into and discharging it from the mixing syringe several times. A small quantity is then drawn up and the tap turned so that the suspension slowly escapes from the side arm adaptor. Three drops, approximately the volume of the dead space in the tap and adaptor, are discarded. A meniscus is left just bulging from the end of the adaptor. The tip of the micrometer syringe is firmly inserted into this and the tap is turned so that the contents of the mixing syringe can be rapidly mixed again with the contents of the reservoir. On drawing up another sample, this is directly transferred to the micrometer syringe which is then detached and returned to its former position ready for implanting. A sterile tube is used to protect the side arm adaptor from contamination between filling operations. The suspension left in the mixing syringe is returned to the cooled reservoir. As the contents of this are used up, air is replaced through a bent hypodermic needle which is stuck through the rubber cap (not shown in Fig. 1). Number 14 needles have been found to be convenient for implanting. A sterile one is used after each refilling; when it has been filled with the contents of the implanting syringe, a little over 0·5 ml. remains, enough to implant 10 mice with single inoculations.

The sterilization and assembly of the device are simple and with a little practice the frequent refilling of the implanting syringe does not constitute a disadvantage from the technical point of view. The errors due to sedimentation are minimized, but not, of course, eliminated by this apparatus. The frequent mixing necessary ensures that the suspension is oxygenated, and this may be an important improvement on the use of a closed syringe as a reservoir, from which the micrometer syringe was filled in earlier experiments. In some of these it was noticed that the number of successful implants fell off somewhat towards the end of an implanting session lasting 2 to 3 hours, and although it cannot be asserted definitely that the use of the improved procedure was directly responsible for removing this source of variation, no such difference in take-rate was found after it had been applied. The whole operation involved in the implantation of 160 mice can be done comfortably within 2 hours, single handed. After a little practice it was found to be



Bennette.

no more difficult to put mice on to a fixed needle than to put the needle into the mice. The section of optical bench on which the stands are mounted is about 14 inches long, so that there is ample clearance between the two pieces for the manipulation of the injections, when the implanting syringe is put into an oblique position and the two stands are placed at each end of the optical bench. To get the necessary rigidity, the base plate is clamped to the edge of a bench during use.

Since the apparatus has proved to be convenient and reliable in practice it will be used for future work and further attention will be directed to the problem of obtaining and handling homogeneous cell suspensions.

SUMMARY.

A device is described which has been designed to facilitate the rapid implantation of up to 200 mice with an accurately uniform dose of homogeneous tumour cell suspensions under aseptic conditions and without the need of assistance.

Thanks are due to Mr. R. L. Warren for his help in making the apparatus.

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THE CARCINOGENIC ACTION OF AMINOSTILBENE DERIVATIVES IN RATS MAINTAINED ON HIGH AND LOW PROTEIN DIETS.

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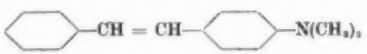
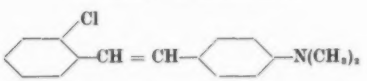
A SERIES of aminostilbene derivatives, many of which showed a considerable inhibitory action on the growth of tumours in rats and mice, has been described by Haddow, Harris, Kon and Roe (1948). Since this type of growth inhibition had previously appeared to be a property of carcinogenic substances, several of the most active of the aminostilbenes were tested for carcinogenic activity. The induction of tumours in rats by 4-amino-, 4-acetamido-, 4-dimethyl-amino, 4-diethylamino- and 2′-methyl-4-dimethylaminostilbene, also by 1-(4′-dimethylamino-phenyl)-2-(1′-naphthyl)ethylene, was recorded. The tumours described included sarcomas, mammary adenomas, cholangiomas, acoustic duct carcinomas and lung adenomas.

In an investigation on the nature of the growth inhibitory action of carcinogenic substances a relation was found between growth inhibition and the protein content of the diet (Elson and Warren, 1947 ; Elson and Haddow, 1947). This effect of diet is particularly well marked in animals treated with aminostilbene derivatives ; a much greater inhibition of both body growth of rats and of growth of Walker Rat Carcinoma 256 being observed in treated rats maintained on a low protein diet than in similarly treated animals maintained on a high protein diet (Elson, 1948).

It was therefore of considerable interest to investigate the effect of diet on the carcinogenic action of aminostilbenes, particularly in view of the well-known effect of diet on the incidence of liver tumours in rats treated with 4-dimethylamino-azobenzene. Two aminostilbenes were chosen for the investigation, 4-dimethyl-aminostilbene (I), which bears a very close resemblance to 4-dimethylaminoazo-benzene, and 2′-chloro-4-dimethylaminostilbene (II), which was found to be less toxic than (I) but also showed a very marked effect of diet in its inhibition of growth of Walker Rat Carcinoma 256.

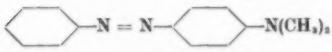


4-Dimethylaminostilbene (I)



2′-Chloro-4-dimethylaminostilbene (II)

* British Empire Cancer Campaign Research Fellow.



4-Dimethylaminoazobenzene.

The induction of tumours by (I) has been described by Haddow, Harris, Kon and Roe (1948). Although tumours in rats had been obtained with (II) (Haddow, private communication) they have not previously been described in print.

EXPERIMENTAL.

Diets.

Diet A (20 *per cent protein*).—This is the high protein diet used in the original work on the effects of diet on the growth inhibitory action of carcinogens (Elson and Warren, 1947). It consists of national wheatmeal flour 72 per cent, casein 11 per cent, milk powder (full cream) 8 per cent, Bemax (Vitamins Ltd.) 2·5 per cent, margarine 3 per cent, salt mixture (Glaxo Laboratories Ltd.) 1 per cent, cod liver oil 1·5 per cent. The average growth rate of young male rats on this diet is about 4 g. per day.

Diet B (5 *per cent protein*).—This low protein diet has been used in most of the experiments on the effect of diet on the inhibition of growth of Walker Rat Carcinoma 256 by carcinogens (Elson and Haddow, 1947 ; Elson, 1948). It consists of starch 85 per cent, casein 5 per cent, Bemax (Vitamins Ltd.) 2·5 per cent, margarine 5 per cent, calcium carbonate 0·5 per cent, salt mixture (Glaxo Laboratories Ltd.) 1 per cent, cod liver oil 1 per cent. The growth rate of young male rats maintained on this diet is usually less than 1 g. per day. The growth of Walker Rat Carcinoma 256, as estimated by the weight of tumours of about 12 days after implantation, in animals maintained on this low protein Diet B is only about 20 per cent less than in rats maintained on the 20 per cent protein Diet A, but 2′-chloro-4-dimethylaminostilbene produces about 90 per cent inhibition of growth of this tumour in animals on Diet B and only about 10 per cent tumour inhibition in those on Diet C.

Diet C (5 *per cent protein* + *methionine*).—This is the same as Diet B, but with addition of 0·4 per cent methionine. The addition of methionine prevents to a large extent the development of fatty livers, which occurs in many of the animals maintained for long periods on Diet B. A 6 per cent protein diet with addition of 0·5 per cent methionine has been found to be very favourable to production of liver tumours by 4-dimethylaminoazobenzene, cholangiomas appearing in as short a time as 2 months and being found in all the animals surviving for 6 months (Elson, 1947). In an experiment carried out by Miss S. Charter, which is still in progress, cholangiomas were found in all animals receiving 4-dimethylaminoazobenzene in Diet C within 5 months, whilst no liver tumours have yet appeared (8 months) in animals receiving the azo compound in Diet A.

Animals and dosage.

Albino rats weighing about 100 to 150 g. at the commencement of the experiment were used.

4-*Dimethylaminostilbene* (I).—Altogether 70 male rats were employed, 20 being maintained on Diet A, 20 on Diet B and 30 on Diet C. The compound was

given by intraperitoneal injection in a first dose of 6 mg. dissolved in 0·5 c.c. arachis oil per rat, followed at intervals of approximately 2 weeks by 4 subsequent doses of 5 mg. in 0·5 c.c. arachis oil. Thus a total dose of 26 mg. per rat was given over a period of 2 months. Administration of the carcinogen was then stopped, and the animals maintained on their respective diets until they died or were killed and examined for tumours.

2′-Chloro-4-dimethylaminostilbene (II).—A total of 60 rats was employed, 10 males and 10 females being maintained on Diet A, the same number on Diet B and 20 males on Diet C. This compound was also administered by intraperitoneal injection, but in this case in doses of 12 mg. dissolved in 0·5 c.c. arachis oil per rat ; 5 doses were given at approximately 2-week intervals making a total of 60 mg. over the period of 2 months.

RESULTS.

4-Dimethylaminostilbene (I).

This compound proved somewhat toxic to the rats maintained on the low protein diets, and in spite of reduction of the dose from 6 mg. to 5 mg. per injection a number of these died within the first few weeks of treatment. These, together with some lost later by cannibalism, have not been included in the autopsy reports. Ignoring these, therefore, 47 rats have been examined post mortem, and among these 34 tumours were found. The type of tumour and the number of days after commencement of treatment with the stilbene until the tumour was identified post mortem are indicated in Table I (Fig. 1). The largest number and greatest variety of tumours were found in the group of animals fed on the high protein Diet A, although none of them was detected before the lapse of more than one year from the commencement of treatment with the carcinogen. The rats in this diet group, however, withstood the toxic action of the dimethylaminostilbene much better than those on the low protein Diet B, and the high incidence of tumours may be ascribed partly to the fact that the animals survived for a longer period. The most interesting result is the large number of cholangiomas obtained in the animals on Diet C. In fact the only tumours appearing in less than one year from the commencement of injection of the carcinogen were 6 cholangiomas, which all appeared in animals on Diet C. Most of these were observed within 5 months of the commencement of treatment, long before any other tumours appeared. All the animals in this diet group which developed tumours had cholangiomas, the only other type of tumour found being a hypernephroma in a rat which also had a cholangioma.

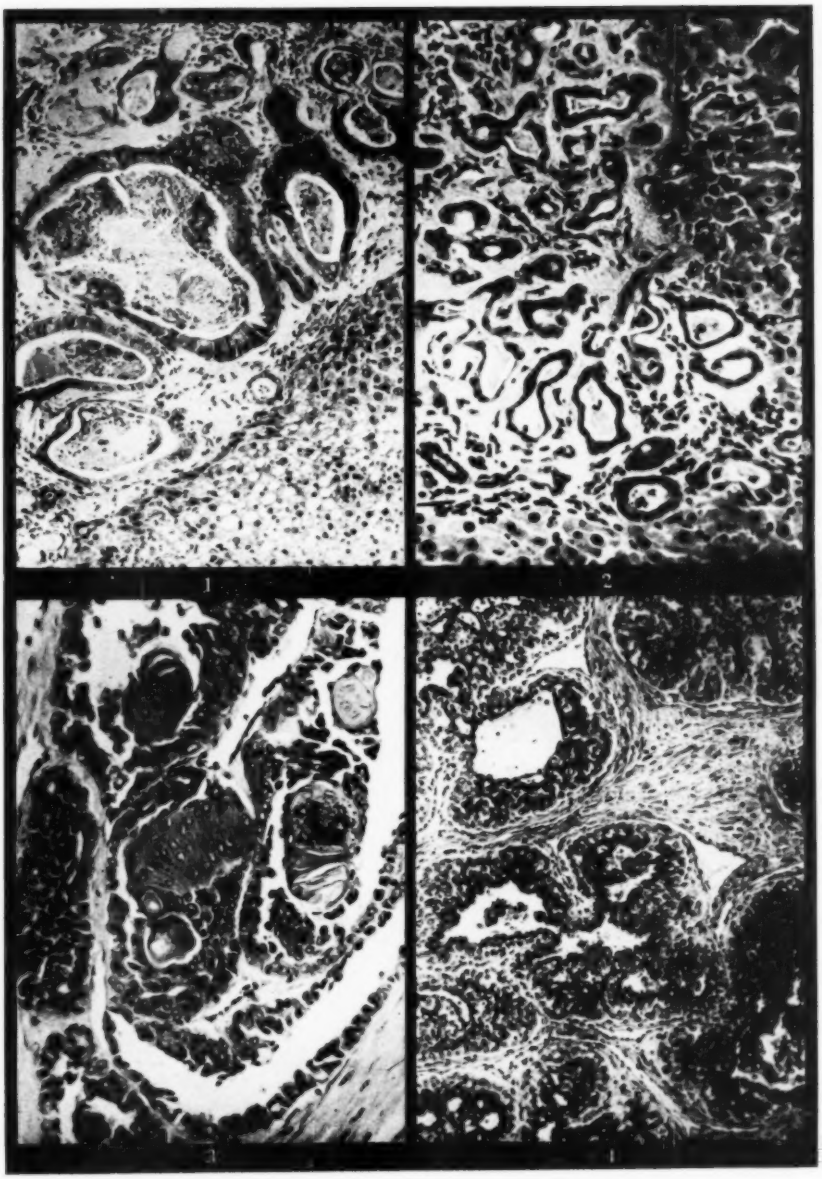
EXPLANATION OF PLATES.

FIG. 1.—Cholangioma in liver of rat treated with 4-dimethylaminostilbene (I), 320 days after first injection. Diet C (5 per cent protein + methionine). Bouin ; H. and E. × 110.

FIG. 2.—Cholangioma in liver of rat treated with 2′-chloro-4-dimethylaminostilbene (II), 285 days after first injection. Diet C (5 per cent protein + methionine). Bouin ; H. and E. × 110. Tumour consists of irregular acinini with fibrous stroma.

FIG. 3.—Mammary carcinoma in female rat treated with 2′-chloro-4-dimethylaminostilbene (II) 411 days after first injection. Diet A (20 per cent protein). Bouin ; H. and E. × 110. Tumour undergoing squamous metaplasia.

FIG. 4.—Adenocarcinoma of mammary gland in male rat treated with 2′-chloro-4-dimethylaminostilbene (II), 534 days after first injection. Diet A (20 per cent protein). Bouin ; H. and E. × 120.



Elson.

TABLE I.—*Tumours Induced by 4-Dimethylaminostilbene (I) in Rats Maintained on High and Low Protein Diets.*

Type of tumour.	Diet.												
	20 per cent protein.					5 per cent protein.		5 per cent protein + methionine.					
Liver :													
Cholangiomas	♂ 366	♂ 467	♂ 512			—		83	86	86	97	145	320	546
Hepatoma			♂ 498											
Kidney :														
Hypernephromas	♂ 467	♂ 512	♂ 597	♂ 659		—					♂ 546			
Lung :														
Adenomas and carcinomas	♂ 441	♂ 468	♂ 597	♂ 659	♂ 663	♂ 534	♂ 663				—			
Pituitary :			♂											
Chromophobe adenoma			597			—					—			
Stomach :			♂											
Carcinoma			468			—					—			
Intestine :														
Squamous-celled carcinomas	♂ 366	♂ 391	♂ 498			—					—			
Thyroid :			♂											
Adenoma			659			—					—			
Skin :														
Squamous-celled carcinoma, fibroma, papilloma	♂ 498	♂ 512	♂ 512			♂ 534					—			
Pancreas :			♂											
Carcinoma (acinar)			512			—					—			
Total number of rats examined post mortem			19			15					13			
Total number of tumours			23			3					8			

The numerals denote the number of days between the first treatment and diagnosis of tumours post mortem.

TABLE II.—*Sites of Occurrence of Multiple Tumours in Rats Treated with 4-Dimethylaminostilbene.*

Sex of rat.	Diet.	Number of days between first treatment and diagnosis of tumours post mortem.	Location and type of tumours.
Male	A	366	Liver (cholangioma). Intestine (squamous-celled carcinoma).
,,	A	468	Lung (bronchogenic carcinoma). Stomach (squamous-celled carcinoma).
,,	A	498	Liver (hepatoma). Intestine (carcinoma). Skin (papilloma).
,,	A	[illegible]	Liver (cholangioma). Kidney (hypernephroma).
,,	C	546	Liver (cholangioma). Kidney (hypernephroma).
,,	A	597	Lung (bronchogenic carcinoma). Kidney (hypernephroma). Pituitary (chromophobe adenoma).
,,	B	534	Lung (bronchogenic carcinoma). Skin (basal-celled carcinoma).
,,	A	512	Liver (cholangioma). Kidney (hypernephroma). Skin (squamous-celled carcinoma). Pancreas (acinar-celled carcinoma).

In a number of animals, particularly those on Diet A, more than one tumour developed in the same rat ; in one case 4 distinct tumours were found. A summary of the type and sites of occurrence of these multiple tumours is given in Table II.

2'-Chloro-4-dimethylaminostilbene (II).

After allowing for early deaths and losses by cannibalism a total of 31 rats treated with this compound were examined post mortem, and 26 tumours were found. The type of tumour and the number of days from the commencement of treatment until the tumour was diagnosed post mortem are given in Table III (Fig. 2, 3 and 4).

TABLE III.—*Tumours Induced by 2'-Chloro-4-Dimethylaminostilbene (II) in Rats Maintained on High and Low Protein Diets.*

Type of tumour.	Diet. 20 per cent protein.	5 per cent protein.	5 per cent protein + methionine.
Liver :			
Cholangiomas	♀ 411 ♂ 534	♀ 534	♂ **285**
Hepatomas	—	♀ **293**	—
Kidney :			
Hypernephromas	—	♀ 610 ♀ 540	—
Lung :			
Adenomas and carcinomas	♀ 392 ♀ 589	—	♂ **285** ♂ 622
Mammary gland :			
Carcinoma	♀ **189** ♀ 386 ♀ 411 ♂ 534 ♀ 562 ♀ 589	♀ **289** ♀ 596	—
Pituitary :			
Chromophobe adenoma	♂ 534	—	—
Stomach :			
Carcinomas	—	♀ 540	♂ 622
Intestine :			
Squamous-celled carcinoma	♀ 562	—	—
Uterus :			
Fibroma and carcinoma	♀ 589	♀ 534	—
Thymus :			
Lymphangioma	♀ 562	—	—
Ear :			
Basal-cell carcinoma	—	♀ **293**	—
Neck :			
Carcinoma	♀ **290**	—	—
Groin :			
Carcinma	♀ 392	—	—
Sub-maxillary gland :			
Carcinoma	♂ 534	—	—
Total number of rats examined post mortem	12	7	12
Total number of tumours	17	9	4

The numerals denote the number of days between the first treatment and diagnosis of tumours post mortem.

A marked difference in behaviour between this compound and 4-dimethylaminostilbene (I) is revealed in that no cholangiomas appeared within 6 months of commencement of treatment in the animals on Diet C as was the case with the latter compound. With Compound (II), apart from the fact that again more

tumours were recorded in the rats on Diet A, which is almost certainly related to the longer survival time of the animals, there appears to be no definite effect of diet on the nature or distribution of the tumours. A number of tumours appeared in less than 12 months, but these were of random distribution and were not confined to any particular type or to animals on a particular diet. The largest number of tumours of one type were mammary carcinomas in females (although one occurred in a male). Since no females were used with compound (I) it is not possible to compare the behaviour of the two stilbenes in this respect.

A summary of the type and sites of occurrence of multiple tumours induced by (II) is given in Table IV.

TABLE IV.—*Sites of Occurrence of Multiple Tumours in Rats Treated with 2′-Chloro-4-Dimethylaminostilbene.*

Sex of rat.	Diet.	Number of days between first treatment and diagnosis of tumours post mortem.	Location and type of tumours.		
Female	B	293	Liver (hepatoma).		Ear (basal-cell carcinoma).
„	B	540	Kidney (carcinoma).		Stomach (squamous-celled carcinoma).
„	A	589	Lung (carcinoma).	Mammary gland (2 carcinomas).	Uterus (carcinoma).
„	B	534	Liver (cholangioma).		Uterus (fibroma).
„	A	562	Intestine (carcinoma).		Mammary gland (2 carcinomas).
„	A	411	Liver (cholangioma).		Mammary gland (2 carcinomas).
„	A	392	Lung (bronchogenic carcinoma).		Groin (carcinoma).
Male	A	534	Liver (cholangioma).	Pituitary (chromophobe adenoma).	Submaxillary gland (carcinoma).

DISCUSSION.

A definite effect of diet on the carcinogenic action of the aminostilbene derivatives investigated is seen in the very short latent period required for the induction of cholangiomas in rats maintained on the diet containing 5 per cent protein with addition of methionine (Diet C). In the group of animals treated with 4-dimethylaminostilbene (I) whilst maintained on this diet, a total of 7 cholagiomas was found, 4 of which appeared within 3 months and 6 before the lapse of 11 months. No other tumours of any type were observed in less than a year from commencement of treatment with this compound in animals maintained on any of the diets.

The low protein Diet C therefore favours the development of cholangiomas, but not of other tumours in rats treated with 4-dimethylaminostilbene (I).

No such effect was observed in the animals treated with 2′-chloro-4-dimethylaminostilbene (II). Only one cholangioma was found in the rats maintained on Diet C, and although this was detected after about 9½ months it was not the earliest tumour to be induced by this compound. A mammary carcinoma was observed after about 6½ months' treatment in the group of rats maintained on the 20 per cent protein Diet A. A carcinoma of the neck in the Diet A animals, a mammary carcinoma, hepatoma and a basal-cell carcinoma of the ear in Diet B

animals, also a lung adenoma and a cholangioma in Diet C animals were all found within about 10 months after commencement of treatment with (II). No preferential effect on any of these diets in reducing the latent period of tumour development in rats treated with 2′-chloro-4-dimethylaminostilbene can therefore be claimed. The behaviour of 4-dimethylaminostilbene in respect of its liver tumour-inducing action is very similar to that of 4-dimethylaminoazobenzene, which it resembles closely in structure. Induction of liver tumours by both compounds is favoured by the same low protein diet, and in both cases it is the latent period required for induction of cholangiomas rather than of hepatomas which is shortened. The diet effect is so marked with 4-dimethylaminoazobenzene that in the experiment referred to previously which is now being carried out by Miss S. Charter, all the animals maintained on Diet C have developed cholangiomas in less than 5 months, whereas no tumours have as yet been detected (8 months) in rats receiving the 20 per cent protein Diet A.

With regard to the general carcinogenic action of the two aminostilbene derivatives (I) and (II), a variety of tumours have been found in 15 different organs. These include most of the types of tumours observed by Haddow, Harris, Kon and Roe (1948), and a number of tumours not previously described. A large number of sarcomas were found by the above authors, who administered the aminostilbenes by subcutaneous injection. No tumour of this type has been observed in the present experiment where the intraperitoneal route of administration was used.

In the paper by Haddow, Harris, Kon and Roe (1948) attention was drawn to the causation by a number of aminostilbene derivatives of haemolymph changes similar to those described by Latnitzki and Woodhouse (1942, 1944) in rats treated with carcinogenic hydrocarbons. The latter authors suggested that the ability of the hydrocarbons to produce such changes might in some way be connected with their carcinogenic action. The similar behaviour of the carcinogenic aminostilbenes has tended to support this suggestion, and Haddow *et al.* (1948) state that although the real significance of the phenomenon of the formation of haemolymph nodes is not at all apparent, it provided an additional reason for investigating the carcinogenic potency of such compounds.

Recent work by the present author may tend to throw considerable light on the significance of these so-called haemolymph changes in relation to carcinogenesis. This work is still in progress and will be fully described later, but it is felt that a brief reference to it in relation to the aminostilbenes should be made here. In studying the toxicity of a series of carcinogenic α ω dimethane sulphonoxyalkanes, it was found that the treated rats develop a general haemorrhagic state and the immediate cause of death is usually a massive haemorrhage in the stomach. With sub-lethal doses it was noted by Miss C. Barrett that the haemorrhagic state could be easily demonstrated by plucking the fur from a small area on the chest of the animal. If positive a number of petechial haemorrhages appear almost immediately. The appearance of stomach haemorrhages and the fact that a marked haemolymph change always accompanies the haemorrhagic state suggested that the haemolymph changes observed by Haddow *et al.* (1948) with aminostilbenes might also be part of a general haemorrhagic state, particularly since some of these compounds also caused haemorrhage in the pyloric region of the stomach. This does appear to be the case, since with a dose of 85 mg./kg. i.p. of 4-dimethylaminostilbene a strongly positive " plucking test " was observed after 8 to 10 days. At post-mortem

examination marked haemolymph changes were observed, and numerous small haemorrhages were evident in many organs, including lungs, testes, seminal vesicles, stomach, intestines, etc.

The haemorrhagic state induced by these compounds is obviously related to their effects on the blood, particularly—as is well known in the case of X radiation—to the diminution in the number of platelets. The haemolymph changes shown by carcinogens if, as appears to be the case with aminostilbene derivatives, they are related in some degree to a more general haemorrhagic state, may represent one aspect of the general growth inhibiting properties of these agents manifested here by prevention of cell division in the normal processes of haemopoiesis.

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CARCINOGENIC ACTIVITY OF METABOLIC PRODUCTS OF 3:4-BENZPYRENE: APPLICATION TO RATS AND MICE.

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NEARLY a quarter of a century has elapsed since the first animal tumours were produced by the application of pure polycyclic aromatic hydrocarbons, and there is still complete ignorance as to the mechanism of local carcinogenic action. It is not known whether it is due to the intact hydrocarbon or to a metabolite, and which of the cell constituents are involved in the process.

Metabolic studies undertaken with a view to obtaining information as to the mechanism of the carcinogenic process have not yet solved the problem. They revealed, however, that the hydrocarbon undergoes metabolic changes, including oxidation to phenolic derivatives, quinones and acids formed by ring-degradation. Only a small fraction is eliminated in the form of unchanged hydrocarbon. The first step in the metabolic chain of events may be the binding of the hydrocarbon by a cell constituent (probably a protein) as suggested by the experiments of Weigert and Mottram (1946*a*, 1946*b*) and Miller (1950, 1951), followed by a local change to an acidic derivative, the nature of which is not yet known, as no pure products have so far been isolated from the metabolites occurring locally. Further changes seem to take place in the liver and during the passage through the intestines (Peacock, 1936; Chalmers and Peacock, 1936, 1941; Weigert and Mottram, 1946*a*, 1946*b*).

The metabolites of carcinogenic hydrocarbons, so far identified, have been isolated from the excreta of animals, but they are present in the circulating blood and may affect the carcinogenic process. It is, therefore, of importance to test the carcinogenic activity of each metabolite which becomes available in the pure state.

Only three phenolic metabolites of carcinogenic hydrocarbons have so far been tested for carcinogenic activity. The two dihydroxy-derivatives of 1:2:5:6-dibenzanthracene proved inactive (Boyland, Levi, Mawson and Roe, 1941; Dobriner, Rhoads and Lavin, 1942; Dunlap and Warren, 1941), while the crude fraction of phenolic metabolites of 3:4-benzpyrene gave one sarcoma in 10 mice tested by the subcutaneous route and one mammary tumour, the spontaneous origin of which could not be excluded (Berenblum and Schoental, 1943).

Since then, 8- and 10-hydroxy-derivatives have been identified as phenolic metabolites of 3:4-benzpyrene in mice, rats, and rabbits (Berenblum and Schoental, 1946), and their structures established by direct comparison of their respective methylation products with the synthetic 8- and 10-methoxy-3:4-benzpyrenes

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(Cook, Ludwiczak and Schoental, 1950). The method of synthesis of 8-hydroxy-3:4-benzpyrene and its methyl ether enabled these pure compounds to be prepared in quantities adequate for biological testing. The results of these tests are now reported, as well as those with 8-methyl-3:4-benzpyrene, which has been synthesised for comparison with the 8-methoxy-compound (Cameron, Cook and Schoental, 1952).

MATERIAL AND METHODS.

Mice were used for the testing of carcinogenic activity of the pure synthetic 8-hydroxy-, 8-methoxy- and 8-methyl-3:4-benzpyrene. 8-Methoxy-benzpyrene was tested also in rats.

The mice were derived from one pair of CBA strain and were bred locally, as were also the rats which were derived from a commercial stock of albino Wistar rats. The animals were housed in metal cages and received rat cake and tap water *ad libitum*.

Freshly prepared solutions (the solvents used were redistilled and freed from fluorescent impurities) of the pure crystalline substances (0·5 per cent solution in acetone in the case of 8-hydroxy-3-4-benzpyrene, a saturated solution in acetone or a 0·5 per cent solution in benzene in the case of 8-methoxy-3:4-benzpyrene) were applied twice a week to the intrascapular region of the skin from a capillary pipette, the hair being removed by clipping with scissors. For subcutaneous injections the pure compounds were dissolved in tricaprylin, and the animals were injected in the left flank, care being taken to prevent leakage of the oily solutions. In the majority of cases the injected oily solution formed a defined nodule palpable under the skin and remaining *in situ* for many weeks or months. The animals were allowed to live as long as possible ; but some were killed when moribund, or when the induced tumours attained excessive size. All the animals were examined *post mortem*, and the tumours and other observed abnormalities confirmed by histological examination. To obtain evidence of the degree of malignancy some of the tumours have been transplanted for several generations into the respective species of animal, and their histological appearance confirmed in the transplants.

RESULTS.

Table I summarizes the results of testing of 8-hydroxy-3:4-benzpyrene. This compound did not produce any local tumours in the 16 mice (10 males and 6 females), into each of which it was injected subcutaneously in two doses, 2 mg. each at 5 months' interval, although 14 animals were alive after 52 weeks, 12 after 79 weeks, and the last 2 were killed when the experiment was terminated 117 weeks after the first injection. Several of the mice exhibited different degrees of leucoses which might have been spontaneous (in the control group of these CBA mice leucoses were also encountered), or accentuated by the action of this compound.

In the animals receiving skin applications of a 0·5 per cent solution of 8-hydroxy-3:4-benzpyrene in acetone (special care was taken to minimise the decomposition of this labile compound by dissolving only very small quantities at a time and keeping the solution in a dark bottle) the treated skin areas remained smooth and soft, and no abnormalities were visible during the first 69 weeks of treatment. However, on continued application small papillomata appeared,

which grew progessively ; five of these became malignant. The experiment was terminated 121 weeks after the beginning of treatment ; about 50 per cent of the animals had some neoplastic local changes.

Table II gives the results of testing 8-methoxy-3:4-benzpyrene in mice and rats. This compound proved to be one of the strongest carcinogens so far known, probably exceeding in potency the parent 3:4-benzpyrene. Thus application of its saturated acetone solution (less than 0·2 per cent) to the skin of mice produced local depilation, dark pigmentation and desquamation a few days after the treatment began ; papillomata arose in multiple foci 10 weeks after the beginning of treatment, so that 50 per cent of the animals had tumours after 14 weeks, and 100 per cent after 22 weeks. Ten mice were killed after 27 weeks of treatment and 9 of these had multiple malignant tumours invading the underlying skeletal muscle ; one had only multiple papillomata. The experiment was terminated after 41 weeks, when the 4 remaining mice with carcinoma were killed. The papillomata from the multiple foci gave in many cases big confluent malignant tumours occupying an area of up to 3 × 1·5 cm. at the site of application.

On subcutaneous injection of 2 mg. in 0·2 ml. of tricaprylin, 8-methoxy-3:4-benzpyrene proved very irritating and ulceration and discharge of the substance occurred in about 4 weeks after the injection ; this effect was more pronounced in the female series. Consequently tumours appeared later among the females (the first tumour appeared after 22 weeks, and 50 per cent of the animals had tumours after 26 weeks) than among the male mice (in which the first tumour appeared after 11 weeks and 50 per cent after 22 weeks). In another experiment 16 mice were injected each with 0·5 mg. of 8-methoxy-3:4-benzpyrene in 0·1 ml. of tricaprylin, but in spite of this reduced dosage some ulceration still occurred. In this series the first tumour appeared 10 weeks after the injection and 50 per cent of the animals had tumours in 14 weeks. However, in both series the extrusion of the material might have been responsible for the non-appearance of tumours in several animals. Still lower dosage would be necessary to obtain truly representative data as to the carcinogenic response of mice. In one case a tumour at the site of a scar appeared as late as 83 weeks after the injection.

No irritation was observed in the rats tested by the subcutaneous route. All the 11 animals developed sarcomata ; the first appeared in 16 weeks and 50 per cent of the rats had tumours 19 weeks after the first injection.

8-Methoxy-3:4-benzpyrene was tested also by skin application in rats. A saturated acetone solution applied twice weekly to the intrascapular region for 39 weeks did not seem to produce macroscopic changes. Subsequently a 0·5 per cent solution of this compound in benzene was applied. The treated area became depilated, scaly, and papillomata started to appear. It is not unlikely that the long induction period of these tumours (about 1 year) might have been shortened if the more concentrated benzene solution had been applied from the beginning of treatment. Once the papillomata appeared they readily became malignant, and keratinous horn-like structures grew to enormous size (5 × 5 × 4·5 cm.) between the ears of the rats. Several of these tumours metastasised to the local nymph nodes. The rat shown in Fig. 1 is a representative example. It shows besides the primary tumour a metastasis which formed a keratinised mass (1·5 × 1·5 cm.) in the submaxillary gland. Two of these tumours were transplanted into weanling rats, and carried successfully through three generations of transplants, which retained the ability to form keratin.

Fig. 1.—Horn-like tumour in a rat at the site of application of 8-methoxy-3:4-benzpyrene and metastasis in the submaxillary gland, 17 months after the first, and 3 months after the last application.

TABLE I.—*Incidence of Tumours in Mice Treated with 8-Hydroxy-3:4-benzpyrene.*

Number and sex.	Dose.	Route.	Time of appearance of tumours. First.	Time of appearance of tumours. 50%.	Duration of experiment.	Number of tumours at site of application.	Other abnormalities.
10 M.	2 mg./0·2 ml. Second dose after 21 weeks	Subcutaneous	—	—	91 weeks	0	1 lymphosarcoma. 1 hyperplasia of lymph nodes.
6 F.	Ditto	„	—	—	117 „	0	3 leucoses. 1 lung adenoma.
10 M.	0·5% in acetone twice weekly	Skin	69 weeks	113 weeks	117 „	3 carcinomas 3 papillomas	4 leucoses. 3 hepatomas. 1 adenocarcinoma of lung.
10 F.	Ditto	„	69 „	113 „	121 „	2 carcinomas 3 papillomas	3 leucoses. 2 lung adenomas.

TABLE II.—*Incidence of Tumours in Mice and Rats Treated with 8-Methoxy-3:4-benzpyrene.*

Animal.	Number and sex.	Dose.	Route.	Time of appearance of tumours. First.	Time of appearance of tumours. 50%.	Duration of experiment.	Number of tumours at site of application.	Other abnormalities.
Rats	11 M.	2 mg./0·2 ml. Second dose after 13 weeks	Subcutaneous	16 weeks	19 weeks	30 weeks	11 sarcomas	—
„	5 F.	Saturated solution in acetone for 39 weeks, then 0·5% in benzene twice weekly	Skin	44 „	50 „	87 „	5 carcinomas	2 metastases in lymph nodes.
„	3 M.	„	„	48 „	48 „	87 „	3 „	1 metastasis in submaxillary gland. 1 metastasis in parotid gland.
Mice	9 M.	2 mg./0·2 ml.	Subcutaneous	11 „	22 „	67 „	9 sarcomas	—
„	12 F.	„ „	„	22 „	26 „	65 „	7 „	—
„	16 F. +M.	0·5 mg./0·1 ml.	„	10 „	14 „	95 „	4 „ 4 „ and carcinomas	5 leucoses. 2 lung adenomas. 1 hepatoma. 1 metastasis in peritoneum and pelvic organs. 2 bronchopneumonias
„	21 F.	Saturated solution in acetone twice weekly	Skin	10 „	14 „	41 „	20 carcinomas 1 papilloma	2 leucoses.

Table III gives the results of subcutaneous testing of 8-methyl-3:4-benzpyrene in mice. At the dosage of 1 mg. in 0·1 ml. tricaprylin this compound produced

TABLE III.—*Incidence of Tumours in Mice Injected Subcutaneously with 8-Methyl-3:4-benzpyrene.*

Number and sex.	Dose.	Time of appearance of tumours.		Duration of experiment.	Number of tumours.
		First.	50%.		
11 M.	1 mg./0·1 ml.	13 weeks	14 weeks	27 weeks	11 sarcomas.
7 F.	ditto	13 „	15 „	27 „	7 „

some local irritation ; the first tumour appeared after 13 weeks, and 50 per cent of the mice had tumours by about 14 weeks after the injections. All the 18 injected mice developed highly malignant tumours invading the adjacent muscle and other tissues. One of these tumours was carried through 3 generations of transplants, retaining its original structure.

DISCUSSION.

The results here reported present several points of interest.

(*a*) It is the first time that carcinogenic activity has been demonstrated by the application to the skin of a phenolic metabolite of a carcinogenic hydrocarbon (pure 8-hydroxy-3:4-benzpyrene) in mice. However, the very long induction period (69 weeks for the appearance of the first papilloma and 113 weeks for tumours to develop in 50 per cent of the animals) corresponds with or rather exceeds the survival times of mice in the carcinogenic tests with the other known metabolites. Hence a low degree of carcinogenic activity such as is exhibited by 8-hydroxy-3:4-benzpyrene may not be as isolated a phenomenon as it seems at present.

No tumours were produced by subcutaneous injection of this compound, which in conjunction with the above results makes it obvious that the carcinogenicity of the parent hydrocarbon is considerably reduced (although not completely abolished) by its metabolic transformation into a phenolic derivative. Of the other benzpyrenols so far tested the 4'- (Cook and de Worms, 1937 ; Shear, 1939), 2- (Dunlap and Warren, 1941) and 5- (Shear and Leiter, 1940) compounds were inactive, while the 6-isomer produced tumours in 2 out of 40 mice when injected subcutaneously (Dunlap and Warren, 1941).

(*b*) Of particular interest is the finding that 8-methoxy-3:4-benzpyrene exhibits a sarcomatogenic and carcinogenic activity of the highest order in both the species tested, mice and rats. While rats are known to develop sarcomata readily on subcutaneous injections of carcinogens, not many skin tumours have been produced in rats by the application of tar or pure carcinogenic hydrocarbons. Berenblum (1949) summarized the relevant literature when he reported on the carcinogenicity of 9:10-dimethyl-1:2-benzanthracene. The latter proved very effective for rat skin, giving rise to 14 tumours (11 of these were malignant) in 20 rats when applied once a week as a 0·5 per cent solution in benzene during 45 weeks.

By comparison with 9:10-dimethyl-1:2-benzanthracene (potency Grade VI, when graded according to the system of Berenblum (1945)), 8-methoxy-3:4-benzpyrene showed towards the skin of rats the potency Grade V, only a little

weaker than the former. However, both compounds were equally effective in their sarcomatogenic action (Grade VII).

8-Methoxy-3:4-benzpyrene proved rather irritating when injected subcutaneously into mice, resembling in this repect 9:10-dimethyl-1:2-benzanthracene and related compounds (Bachmann, Kennaway and Kennaway, 1938). The tissues sloughed off 4 to 5 weeks after the injection, and most of the compound was discharged. Thus in mice the results of subcutaneous injection are probably not truly representative, and more reliable data could be obtained with a lower dosage. In the two experiments with this compound using 2 mg./0·2 ml. and 0·5 mg./0·1 ml. per mouse respectively, tumours appeared earlier following the lower dosage, but even the latter was too irritating and many animals remained with scars, tumour-free, for many months.

The outstanding carcinogenic activity of 8-methoxy-3:4-benzpyrene is not shared by the other two isomers so far tested, namely, 4′- and 5-methoxy-3:4-benzpyrenes (Cook and de Worms, 1937 ; Shear, 1939 ; Berenblum and Schoental, 1944), nor by 4′-methoxy-9:10-dimethyl-1:2:benzanthracene, considered to be the methylation product of the phenolic rat metabolite of the highly carcinogenic 9:10-dimethyl-1:2-benzanthracene (Dickens, 1946). With the exception of 5-methoxy-3:4-benzpyrene which produced sarcomata in 2 out of 10 mice, these were inactive.

Carcinogenic activity seems more general among the methyl derivatives of 3:4-benzpyrene. Including the active 8-methyl compound for which results are recorded in Table III, data are now available for eight of the twelve theoretically possible monomethylbenzpyrenes (Schürch and Winterstein, 1935 ; Shear, 1939 ; Shear and Leiter, 1940 ; Dunlap and Warren, 1941 ; Dunlap and Warren, quoted by Fieser and Heymann, 1941 ; Dunlap, quoted by Jones, 1942). Of these only two have proved inactive (the 2′- and 3′- methyl compounds). It is evident from a survey of the data that, although 8-methyl- and 8-methoxy-3:4-benzpyrene are both strongly carcinogenic, no general parallelism exists in the carcinogenic response of other methyl and methoxy derivatives of benzpyrene. For instance, both 4′-methyl- and 5-methyl-3:4-benzpyrene gave tumours in a very high proportion of the treated mice although, as stated above, the 4′-methoxy compound was inactive and the 5-methoxy compound feebly active.

A similar lack of parallelism is shown by methyl- and methoxy-derivatives of other polycyclic aromatic hydrocarbons (compare 9:10-dimethyl- and 9:10-dimethoxy-1:2-benzanthracene ; although the former is a very potent carcinogen, the latter failed to produce tumours in 15 months after subcutaneous injection of 2 mg. into each of 10 mice (Berenblum and Schoental, 1944)).

The fact that 8-methyl-3:4-benzpyrene is an effective carcinogen discounts the suggestion of Greenwood (1951), who explained the inactivity of 4′-methyl-1:2-benzanthracene by postulating that a methyl substituent at the metabolically attacked position may interfere with the metabolic process and thus destroy the potential activity of the compound.

SUMMARY.

Pure synthetic 8-hydroxy, 8-methoxy- and 8-methyl-3:4-benzpyrene have been tested for carcinogenic activity with the following results :

(*a*) 8-Hydroxy-3:4-benzpyrene (a phenolic metabolite of 3:4-benzpyrene) produced tumours after more than 69 weeks of application to the skin of mice,

but none by the subcutaneous route. Thus its carcinogenic activity is rather feeble.

(*b*) 8-Methoxy-3:4-benzpyrene produced sarcomata readily in mice and rats when injected subcutaneously. It also produced tumours not only in all the mice but also in the skin of all the rats to which it was applied. The high malignancy of the skin tumours in the rats was indicated by the presence of secondary keratinizing carcinomata in the lymphoid tissues, and by the growth of transplants in weanling rats. Thus, this methyl ether of a metabolite of 3:4-benzpyrene ranks among the most potent carcinogens so far known for mice and rats.

(*c*) 8-Methyl-3:4-benzpyrene tested by the subcutaneous route in mice produced in all the animals tested highly malignant haemorrhagic tumours invading the adjacent muscle and other tissues. The potency of this methyl-derivative is at least equal to that of 3:4-benzpyrene itself; tumours developed very quickly, 50 per cent of the animals having tumours in 15 weeks.

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CARCINOGENIC ACTIVITY OF METABOLIC PRODUCTS OF 3:4-BENZPYRENE: PATHOLOGICAL STUDIES.

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WE have made a comparative study of the lesions in rats and mice treated with derivatives of 3:4-benzpyrene described in the preceding paper, and of the changes in mice treated in 1937 with a crude sample of the metabolite of 3:4-benzpyrene prepared from the bile of rabbits after intravenous injection of the hydrocarbon in colloidal suspension.

The chemical identity of the metabolite (" BPX ") used in the earlier experiments was not then known, but later work by Chalmers and Crowfoot (1941) and by Berenblum and Schoental (1943) indicated that it was probably 8-hydroxybenzpyrene. Technical difficulty of preparing this substance from natural sources in sufficient quantity for bioassay probably accounts for the paucity of information about it. Nevertheless Berenblum and Schoental (1943) recorded in 1 of 3 mice surviving 10 months after a single subcutaneous injection " a small nodule attached to the thin capsule around the injected material, which proved histologically to be a spindle cell sarcoma ". The lesion was not illustrated.

Our original sample of " BPX " was injected intradermally into the interscapular region of 11 mice on a single occasion, and into 5 of them again 8 days later and into 2 of these after a further 7 days. Six of these mice survived for more than a year from the start of the experiment and died or were killed, one each on the 391st, 426th, 439th, 468th day, and 2 on the 505th day of experiment. In 2 of these mice (468th and 505th day) the skin was thickened and showed histological evidence of hyperplasia. This was of unspecific type such as is seen in many chronic or healing lesions, and was possibly due to infestation with lice and ringworm which was present in these mice. Pullinger (1940) described thickened, hyperkeratotic squamous epithelium in old mice where ulceration due to lice bites was widespread.

In view of the present observations of the effects of the synthetic compound it is probable that the dosage of the natural metabolite was below that necessary to produce lesions with this relatively weak carcinogen, and further investigations seem justified.

Post-mortem examinations were carried out on most of the animals treated with pure synthetic 8-hydroxy-, 8-methoxy- and 8-methyl-3:4-benzpyrene, and all gross or suspicious lesions were examined histologically.

The following cases are selected to show the general range of histological evidence on which these new substances are classed as carcinogens. Of the 20

* Formerly the Glasgow Royal Cancer Hospital.

mice painted with synthetic 8-hydroxybenzpyrene, 6 showed well differentiated squamous papillomata and 5 highly keratinizing squamous carcinomata in which many cell nests were present and mitoses were common.

In 7 mice leucoses were present and in 2 there were lung adenomata. In one male mouse two ulcerated nodules were seen in the left triangle of the neck which appeared to be lymph nodes. Two or three small nodules were also present in the deep subcutaneous tissue over the nape of the neck. Nodules were also noted in both lungs, kidneys and the heart and a large mass in the mesentery. On histological examination these were all seen to be papillary adenocarcinomata, the origin of which appeared to be in the lung. A hepatoma was also present in this mouse and in 2 other mice of this group.

No tumours were found at the site of inoculation in 16 mice receiving subcutaneous injections of 8-hydroxy-3:4-benzpyrene but 3 of these had leucosis, one hyperplastic lymph nodes, one a lymphosarcoma and one a lung adenoma.

Mouse Female 90/52 was killed 824 days after first skin painting with 8-hydroxy-3:4-benzpyrene. At post-mortem examination a large horny tumour was present between the ears. No abnormalities were noted elsewhere.

Histologically the tumour was seen to be a squamous papilloma showing much hyperkeratosis and cell-nest formation. A transition to squamous carcinoma was present at the base of the papilloma and could be seen infiltrating deeply under the surrounding normal skin (Fig. 1).

All of the 21 mice painted with 8-methoxy-3:4-benzpyrene showed lesions in the skin, and on histological examination 20 of these were seen to be well differentiated squamous carcinomata and one was a squamous papilloma. In 2 animals leucosis was noted.

Mouse Female 67/51 died 294 days after first skin painting with 8-methoxy-3:4-benzpyrene. At post-mortem examination large tumours were noted on the nape of the neck. The submaxillary and axillary lymph nodes were enlarged. The liver and spleen showed multiple pale depressed areas. The other organs showed no abnormalities.

Histological examination showed a well differentiated squamous papilloma with transition to squamous carcinoma which spread deeply into the subcutaneous tissue. Many cell nests were present and mitoses were fairly common (Fig. 2). The lesions in the lymph nodes, liver and spleen were leukaemic.

Histological examination of the 24 tumours produced in the 37 mice injected subcutaneously with 8-methoxy-3:4-benzpyrene showed mostly spindle-celled fibrosarcomata. In 4 cases the tumour was of mixed type, partly sarcomatous and partly carcinomatous, and in 3 mice it appeared to be of muscle origin. In 5 animals leucoses were present, in 2 lung adenomata and in 1 a hepatoma.

Mouse Female 341/51 was killed 407 days after subcutaneous injection of 8-methoxy-3:4-benzpyrene. At postmortem examination a huge nodular ulcerated tumour was found involving the skin, subcutaneous tissues and abdominal wall. It was adherent to the kidney and invading the suprarenal.

Histologically it was seen to be a spindle-celled sarcoma showing some pleomorphism. There was direct spread of the tumour through the abdominal wall to the suprarenal, which was entirely replaced by new growth. The kidney was not invaded, though closely invested by tumour.

The 8 rats painted with 8-methoxy-3:4-benzpyrene all developed huge keratinizing squamous carcinomata which invaded deeply into the underlying

tissues. Many cell nests were present and mitoses were frequent. In 2 cases there were metastases in the regional lymph nodes and in 2 the parotid and submaxillary glands were involved. One tumour was transplanted and grew rapidly ; it preserved the same histological appearances as the original tumour.

Rat Female 628/51 was killed 522 days after skin painting with 8-methoxy-3:4-benzpyrene was begun and was a typical example of the lesions met with in this group. There was a large keratinized tumour at the site of painting on the nape of the neck, measuring $5 \times 5 \times 4{\cdot}5$ cm., spreading up between the ears and invading the eye. A secondary, highly keratinized tumour was present in a cervical lymph node, measuring $1 \times 1 \times 1{\cdot}5$ cm. No abnormalities were detected in the internal organs.

On histological examination there was a well differentiated keratinizing squamous carcinoma in which mitotic figures were frequent (Fig. 4). The regional lymph node was seen to be almost completely replaced by a keratinizing squamous carcinoma. The centre of the tumour was composed of keratinized material (Fig. 5).

Rat 705/51 was killed 28 days after part of the tumour from Rat 628/51 had been transplanted subcutaneously. On post-mortem examination a tumour was seen at the site of transplantation, which was histologically a highly keratinizing squamous carcinoma. Mitoses were numerous, and the tumour was actively invading the fibrous tissue covering it (Fig. 6).

Of the 11 rats injected subcutaneously with 8-methoxy-3:4-benzpyrene, 8 developed fibrosarcomata at the site of injection. Giant cells with one or more nuclei were present in 5 cases. In 3 tumours there were areas with long spindle-shaped giant cells where the appearances were those of a muscle tumour.

Sections were stained with phosphotungstic acid haematoxylin, Heidenhain's iron haematoxylin and celestin blue in an attempt to show cross striation, but this was not observed. In sections stained by van Gieson's method the tumour cells stained yellow.

Rat Female 773/50 was killed 179 days after subcutaneous injection of 8-methoxy-3:4-benzpyrene. A large tumour was present at the site of inoculation which showed the structure of a fibrosarcoma on histological examination (Fig. 7). It was adherent to the overlying skin, which was ulcerated.

All the mice (11 male and 7 female) injected subcutaneously with 8-methyl-3:4-benzpyrene developed large haemorrhagic tumours from 2 to 4 cm. in diameter. These were adherent to the skin and deeply infiltrating the underlying muscle. In 2 female mice a large abdominal tumour was present. It was adherent to the spinal column, liver, spleen and kidneys.

On histological examination it was seen to be invading the kidneys. A similar tumour was found in one male mouse and in another secondary deposits were seen in the diaphragm.

These tumours were sarcomata of spindle-cell type in which mitoses were numerous. Areas of haemorrhage and degeneration were present in them all. In 5 cases there were areas where the appearance was that of a myoblastoma. Long spindle cells were seen with several nuclei lying in the long axis of the cell. In one case cross-striation was seen at the tapering ends of the giant spindle cells.

Mouse Male 204/52 was killed 154 days after subcutaneous injection of 8-methyl-3:4-benzpyrene. At post-mortem examination a large tumour $2 \times 1{\cdot}5$ cm. was found. No abnormalities were seen in the internal organs.

Histologically this was seen to be a spindle-celled sarcoma in which mitoses were frequent. It was infiltrating the underlying muscle (Fig. 8).

DISCUSSION.

It soon became obvious that, despite their close chemical similarity, there was a striking difference between the carcinogenicity of 8-hydroxy- and 8-methoxy-3:4-benzpyrene, as shown by the longer latent period and lower incidence of tumours after treatment with the former compound. Nevertheless the skin tumours caused by these two substances showed a similar range of histological variation between keratinizing papilloma and epidermoid carcinoma, though the proportion of malignant tumours was lower in the 8-hydroxy-3:4-benzpyrene group. The sarcomata caused by 8-methoxy- and 8-methyl-3:4-benzpyrene could not be distinguished from each other by histological examination. We reached this conclusion by grading independently all the lesions in order of malignancy without knowledge of their source, and with only the very slight differences of opinion which are bound to occur in such a method.

It is evident therefore that the longer latent period and lower incidence of tumours in mice painted with 8-hydroxy- than with 8-methoxy-3:4-benzpyrene are not necessarily associated with a lesser degree of malignancy or different histogenesis in such tumours as are induced. This is in keeping with the general experience of chemical carcinogenesis.

The leucoses, lung adenomata and hepatomata noted amongst these mice have also been seen frequently in control groups of CBA mice.

We should like to thank Dr. B. D. Pullinger for her help in the examination of material from several animals in our absence, and for giving us the benefit of her experience in some of the cases where the histogenesis of tumours was in doubt.

DESCRIPTION OF PLATES.

FIG. 1.—90/52. Squamous carcinoma in skin of female mouse 824 days after first painting with 8-hydroxy-3:4-benzpyrene. H. and E. × 130.

FIG. 2.—67/51. Squamous carcinoma in skin of female mouse 294 days after first painting with 8-methoxy-3:4-benzpyrene. H. and E. × 120.

FIG. 3.—341/51. Sarcoma in mouse 407 days after subcutaneous injection of 8-methoxy-3:4-benzpyrene. H. and E. × 480.

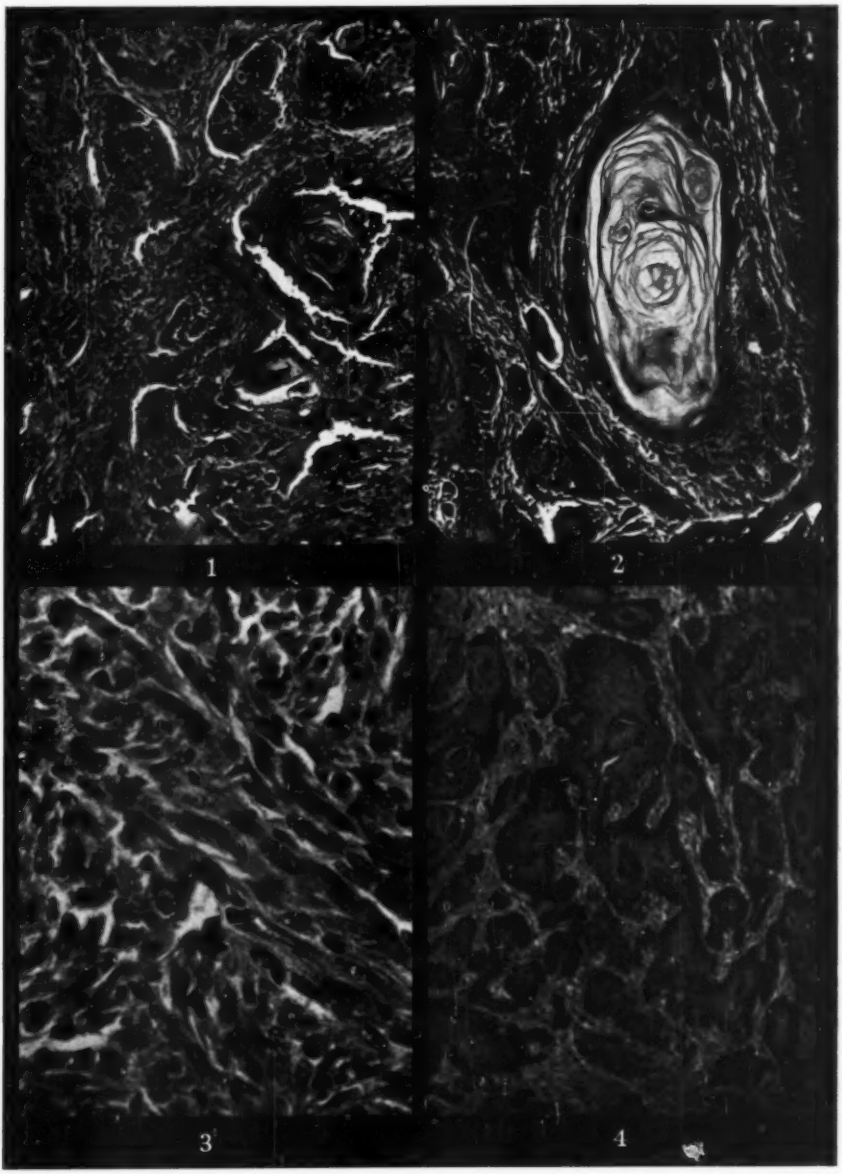
FIG. 4.—628/51. Squamous carcinoma in skin of rat 522 days after first painting with 8-methoxy-3:4-benzpyrene. H. and E. × 70.

FIG. 5.—628/51. Squamous carcinoma showing secondary invasion of regional lymph node from same rat as Fig. 4. H. and E. × 80.

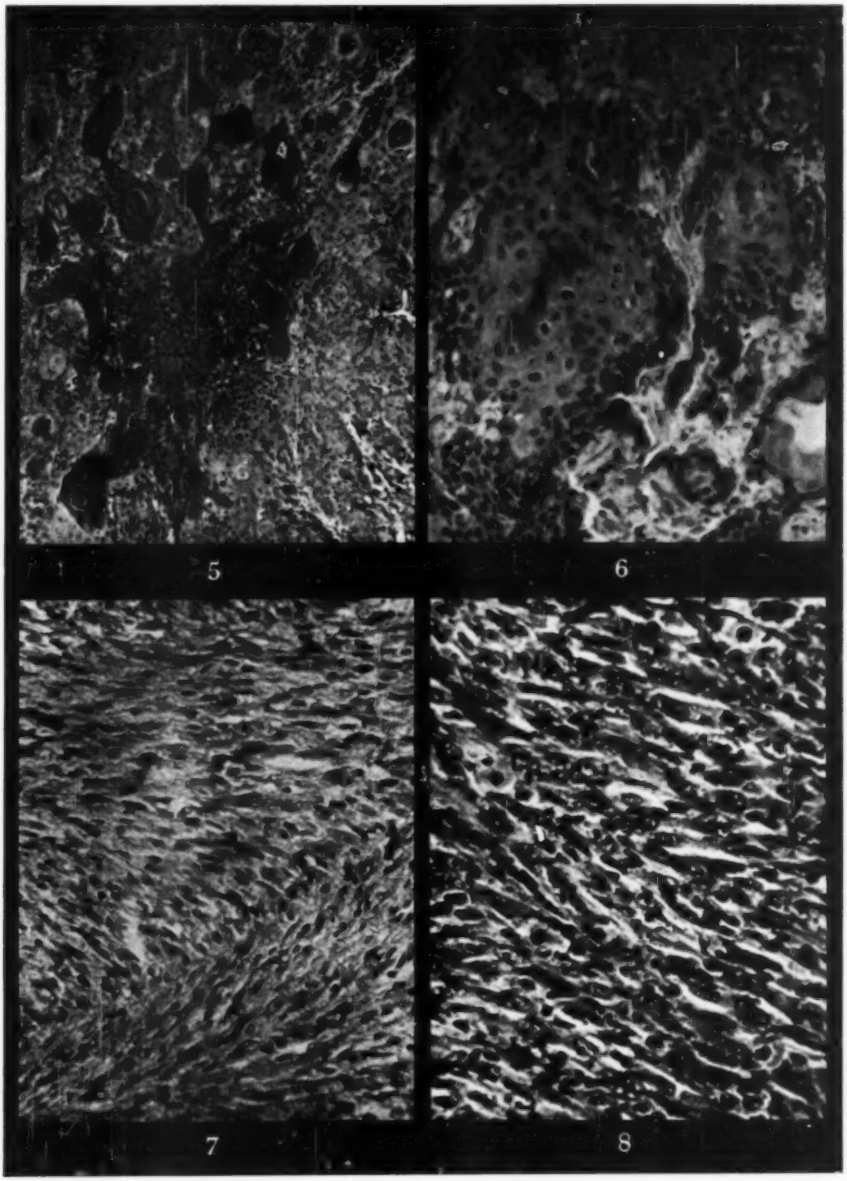
FIG. 6.—705/51. Transplanted squamous carcinoma in rat from case 628/51. H. and E. × 190.

FIG. 7.—773/50. Fibrosarcoma in rat after subcutaneous injection of 8-methoxy-3:4-benzpyrene. H. and E. × 225.

FIG. 8.—204/52. Sarcoma in mouse after subcutaneous injection of 8-methyl-3:4-benzpyrene. Iron haematoxylin and van Gieson. × 350.



Peacock and Head.



Peacock and Head.

SUMMARY.

The lesions induced by 8-hydroxy-, 8-methoxy- and 8-methyl-3:4-benzpyrene include—

(i) Epithelial tumours ranging from well differentiated keratinizing papillomata to anaplastic epidermoid carcinomata.

(ii) Mesoblastic tumours including myoblastoma and undifferentiated spindle-celled sarcomata.

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THE CARCINOGENIC PROPERTIES OF 2-AMINO-1-NAPHTHOL HYDROCHLORIDE AND ITS PARENT AMINE 2-NAPHTHYLAMINE.

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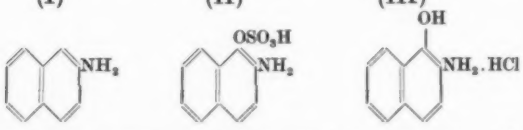
IN 1951, Bonser, Clayson and Jull published some of the results of a quantitative study of the metabolism of 2-naphthylamine by various species. An apparent correlation was demonstrated between the proportion of a dose of 2-naphthylamine (I) excreted by way of the urine as 2-amino-1-naphthol derivatives (II) and the biological response of the species to treatment with 2-naphthylamine. Thus it was shown that the dog, which is particularly susceptible to 2-naph-

(I) (II) (III)



thylamine carcinogenesis, excretes 55 to 70 per cent of a dose of 2-naphthylamine as 2-amino-1-naphthol conjugates, whereas the mouse, rat and rabbit, which are less susceptible, excrete smaller quantities in this form. Quantitative studies also revealed that the concentration of 2-amino-1-naphthol derivatives in the urine relative to the plasma was approximately 200:1 and from this it was concluded that the exposure of the urinary tract epithelium to the metabolite was very much greater than that of any other part of the body.

Evidence was also presented (Bonser *et al.*, 1951) that synthetic 2-amino-1-naphthol hydrochloride (III) was a carcinogen, using the method devised by Jull (1951) of surgical introduction of paraffin wax pellets containing the chemical into the lumen of the bladder of the mouse. The present communication contains the results of an extended series of experiments which were undertaken to test the carcinogenic potency of the parent amine and of its metabolite by this method. The results of other experiments previously conducted and designed to discover a small experimental animal susceptible to bladder carcinogenesis by 2-naphthylamine are also reviewed.

METHODS.

Experiment 1.

Surgical introduction of wax pellets into the bladders of mice.

White mice obtained from a dealer were used. The standard pellet weighed 10 to 20 mg. and contained 10 to 15 per cent by weight of the suspected carcinogen

in suspension in 56° or 80° melting-point paraffin wax (i.e., approximately 1 to 2 mg. of carcinogen per pellet). The technical procedures are described by Jull (1951). It was found convenient to distend the bladder at *post mortem* with fixative (Bouin's solution), then to bisect the organ in the saggital plane and to examine the interior with a hand lens or dissecting microscope. By this means tumours could be detected not infrequently. The two halves of the bladder were then embedded on the cut surface. As the work progressed and it was found that tumours did not occur on the suture line of the dome, this portion was cut away before embedding took place (Fig. 5), as cutting the silk sutures tended to tear the sections.

The results of such implantations are shown in Fig. 1 to 4. Experience has shown that epithelial hyperplasia is to be expected even following the implantation of an innocuous pellet such as paraffin wax and therefore this change is not

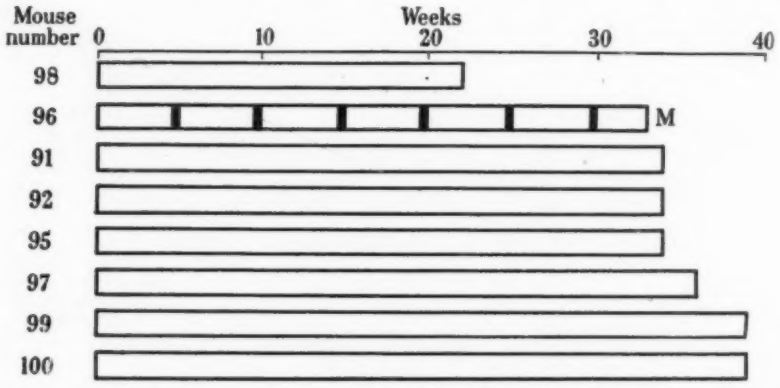


FIG. 1.—Results of implantation of pellets containing 2-naphthylamine in 8 mice which survived for 20 weeks or longer and did not develop tumours. M = metaplasia.

recorded in the charts. Such hyperplasia tends to reach a maximum between 12 and 20 weeks and to subside in the later stages of the experiment (Fig. 6 and 7). Complications such as leakage of urine, infection of the urinary tract, formation of concretions and blocking of the urethra by the pellet occur. It has not been possible to estimate the mortality from these causes with any degree of accuracy owing to an outbreak of ectromelia, but in spite of this, sufficient mice have survived for 25 weeks or longer to show that the method is practicable and valuable.

2-*Naphthylamine.*—The purified chemical (Bonser, 1943) was used. In 8 mice, one surviving for 22 weeks and 7 surviving from 33 to 39 weeks, no tumours were seen (Fig. 1 and 9). In Mouse 96 (33 weeks) there was generalised epithelial hyperplasia and a localised area of squamous metaplasia high up on the wall of the bladder.

2-*Amino*-1-*naphthol hydrochloride.*—The chemical was synthesised as described by Bonser *et al.* (1951), except in the case of the pellets introduced into Mice 256, 109 and 292, when it was extracted from the urine of a dog fed with 2-naphthylamine (*in vivo* material). Six mice survived from 22 to 28 weeks, and no tumours were seen (Fig. 2). Of 8 mice which survived from 30 to 39 weeks, the epithelium was

normal in one (33 weeks); one showed extensive squamous metaplasia at 35 weeks (Mouse 23); in one there was an adenoma at 30 weeks (Mouse 109); and in the remaining 5 mice, carcinomas, 4 of which had progressed to the invasion of muscle, were found (Fig. 12 to 15). In 3 of these mice papillomas were also present and in 2 metaplasias, that in Mouse 76 being of squamous and mucous type (Fig. 11).

Paraffin wax.—In this control series standard-sized pellets were made from

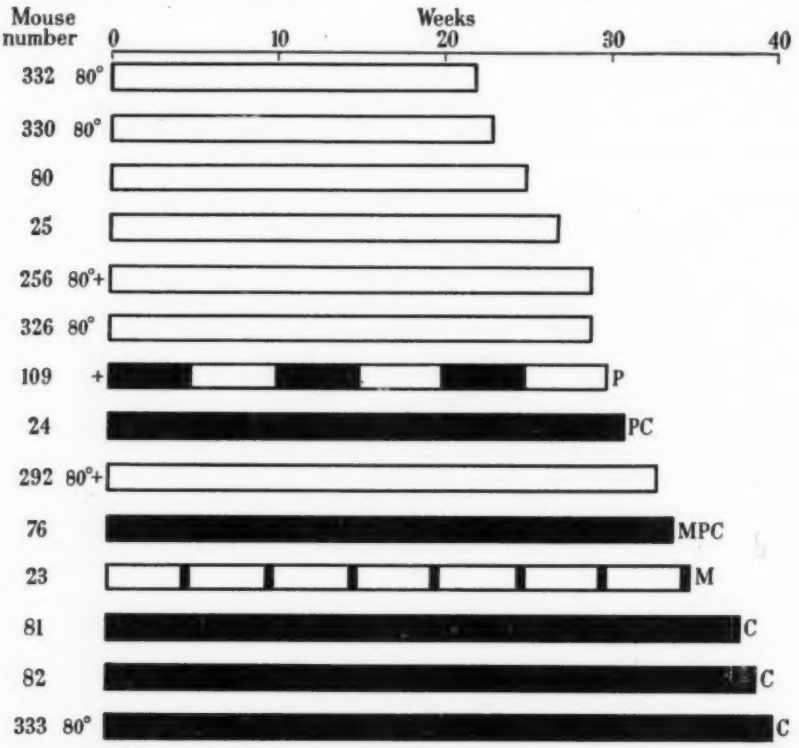


FIG. 2.—Results of implantation of pellets containing 2-amino-1-naphthol hydrochloride in 12 mice which survived for 20 weeks or longer. M = metaplasia; P = papilloma or adenoma; C = carcinoma; 80° = pellet made of wax M.P. 80°; + = *in vivo* material.

wax only, except in the case of Mice 85 and 86, when 10 to 15 per cent of ammonium chloride was incorporated in the pellets. Seven mice survived 21 to 30 weeks and 5 mice 30 to 39 weeks (Fig. 3). No tumours or metaplasia were observed (Fig. 8).

3:4:5:6:*Dibenzcarbazole.*—It was suggested by Armstrong and Bonser (1950) that wherever this compound could be brought into contact with the tissues in adequate concentration, carcinogenic action might be anticipated. It was therefore decided to test a sample kindly supplied by Professor E. Boyland of the Chester Beatty Research Institute, Royal Cancer Hospital, London. Eight mice

survived for 14 weeks or longer (Fig. 4). The first papilloma appeared at 14 weeks, which was earlier by 16 weeks than any tumour caused by 2-amino-1-naphthol hydrochloride. The first carcinoma occurred at 17 weeks (Fig. 16 and 17). This was earlier by 14 weeks than the first 2-amino-1-naphthol carcinoma and by 8 weeks than the methylcholanthrene-induced tumour described by Jull (1951). Squamous metaplasia was a marked feature in 5 mice.

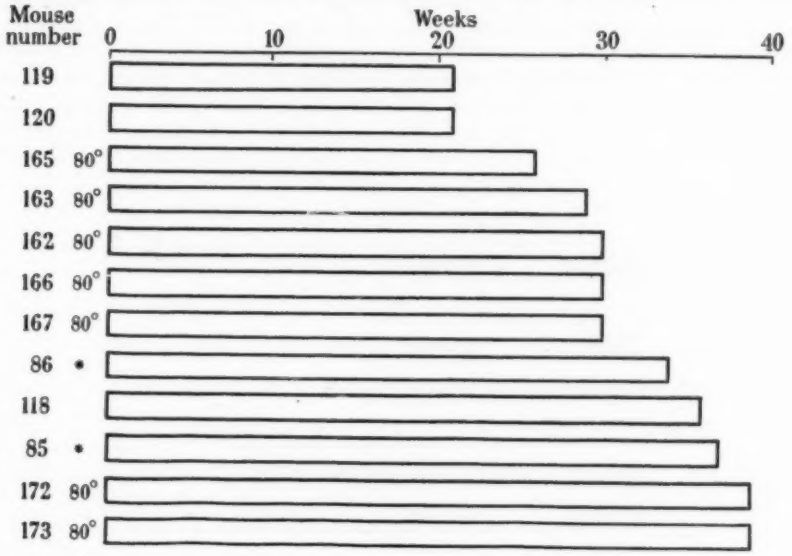


FIG. 3.—Results of implantation of paraffin wax pellets in 12 mice which survived for 20 weeks or longer and did not develop tumours. 80° = pellet made of wax M.P. 80°; * = NH_4Cl in pellet.

Experiment 2.

Feeding of mice, rats and rabbits.

(a) 2-*Naphthylamine* by stomach-tube to IF mice fed on a mixed diet, each mouse weighing on an average 25 g. (Table I): The maximum tolerated dose was given and treatment was continued until the mice died. The only pathological change attributable to the chemical was extensive but somewhat focal proliferation

TABLE I.—*Administration by Stomach-tube to IF Mice of 5 mg. 2-Naphthylamine in Arachis Oil (twice weekly) ≡ 400 mg. per kg. Body-weight per week.*

	Total mice.		Weeks of treatment.				Cholangiomas.		
	F.	M.	30–39.	40–49.	50–59.	60–72.	F.	M.	%.
2-naphthylamine	12	13	$\frac{0}{3}$	$\frac{0}{8}$	$\frac{2}{5}$	$\frac{8}{9}$	5	5	40·0
Arachis oil	5	6	0	$\frac{0}{7}$	$\frac{0}{4}$	0	0	0	0

Numerator = mice with cholangiomas. Denominator = mice dying within the period stated.

of the small intrahepatic bile ducts, associated with mild portal cirrhosis (Fig. 18). This was termed "cholangioma" by Cook, Hewett, Kennaway and Kennaway (1940), denoting a proliferative process which may proceed to neoplasia. This change was not observed in 11 control mice which died before the end of the 59th week nor in breeding mice of the same strain observed for many years.

(b) 2-*Naphthylamine* by stomach-tube to CBA mice fed on a mixed diet, each mouse weighing on an average 30 to 35 g. (Table II): Again the only pathological changes were in the liver. Half of the experimental mice which survived for 50 weeks developed hepatomas, which were regarded as true tumours. Many grew to a large size, and became pedunculated and then infarcted. Two tumours were histologically malignant. Hepatomas did not occur in a small group of control mice surviving as long as the experimental animals, but benign tumours were present in control breeding mice to the extent of 8·1 per cent (Table II). Although

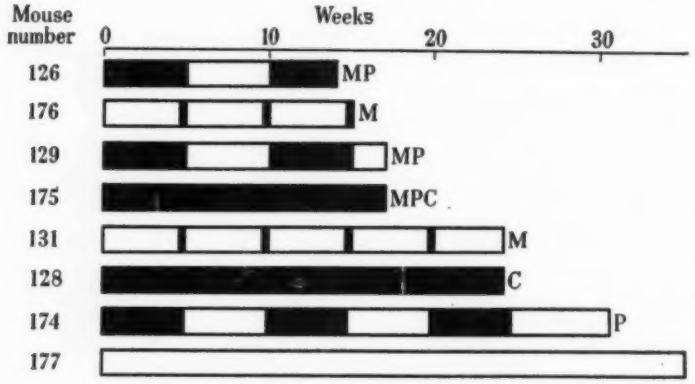


FIG. 4.—Results of implantation of pellets containing 3:4:5:6-dibenzcarbazole in 8 mice which survived for 14 weeks or longer. M = metaplasia; P = papilloma or adenoma; C = carcinoma.

Andervont (1950), has pointed out that variations in incidence of hepatomas must be regarded with caution, this difference would appear to be biologically as well as statistically significant.

TABLE II.—*Administration by Stomach-tube to CBA Mice of 5 mg. 2-Naphthylamine in Arachis Oil (twice weekly) ≡ 240 mg. per kg. body-weight per week.*

	Total mice.		Weeks of treatment.					Hepatomas.		
	F.	M.	30–39.	40–49.	50–59.	60–69.	70–89.	F.	M.	%.
2-naphthylamine	14	9	0	0/2	1/4	2/3	10/14	7	6	52·0
Arachis oil	7	7	0/2	0/5	0	0	1*/7	0	0	0
Breeding	102	46	0	0/16	1/40	0/20	11/72	7	5	8·1

* Single cholangioma.

Numerator = mice with hepatomas. Denominator = mice dying within the period stated.

(c) 2-*Naphthylamine* by incorporation in the diet of CBA mice: The basic diet consisted of casein (20 per cent), starch (50 per cent), arachis oil (10 per cent), yeast (10 per cent), vitamin oil, salts and water to make a paste. The chemical was dissolved in arachis oil and well mixed with the diet. Modifications of the diet were also arranged so that the fat content was high (30 per cent arachis oil), excess of cystine was added (0·5 per cent) or the protein content was reduced (10 per cent casein) and cystine added (0·5 per cent). The amount of the diet eaten on an average by each mouse was weighed, and it was found that only 160 mg. of chemical per kg. of body weight was ingested compared with 240 to 400 mg. by stomach tube (Tables I, II and III). Hepatomas occurred in each group (Table III), the variations in incidence not being significant. Malignant hepatomas were found in 16 mice, one having metastasised to the lungs.

TABLE III.—*Feeding of CBA Mice with 2-Naphthylamine in Adequate Synthetic Diet ≡ approx. 160 mg. per kg. body-weight per week.*

Diet.	Total mice.		Weeks of treatment.					Hepatomas.		
	F.	M.	40–49.	50–59.	60–69.	70–79.	80–89.	F.	M.	%
Basic (20% protein)	12	14	0	0/3	1/1	2/2	8/20	5	6	42·3
High fat (30%)	12	14	0	1/3	0	0	13*/23	9	5	53·6
High cystine (0·5%)	15	14	0/1	1/1	3/3	9/24	0	4	9	48·0
Low protein (10%) + High cystine (0·5%)	15	15	1/2	1/3	0	8/24	1/1	7	4	36·7

* Metastasis to the lungs in one mouse.

Numerator = mice with hepatomas. Denominator = mice dying within the period stated.

(d) 2-*Naphthylamine* by incorporation in the diet of rats: Albino rats were supplied by Glaxo Ltd. and were three-quarters grown. The basic diet consisted of casein (10 per cent), starch (80 per cent), arachis oil (5 per cent), yeast (2·5 per cent), vitamin oil and salts. To this was added 2-naphthylamine (0·1 g. per kg.) and water to make a stiff dough. From Table IV it is seen that 4 papillomas of the bladder occurred among 31 rats which survived treatment for 60 weeks or more; no tumours occurred in the controls. Reduction in the protein content of the diet may have played some part in the appearance of the tumours but the numbers are too small to draw any firm conclusion. Hyperplasia of the bladder epithelium was seen in 6 treated and 5 control rats, and squamous metaplasia in 3 and 5 rats respectively. The nematode *Trichosomoides crassicauda* was seen in the bladder epithelium or free in the lumen of 7 control rats but not in treated animals. No cellular reaction was seen in relation to them, which was the experience of Spitz, Maguigan and Dobriner (1950). The liver changes were of very mild type. Twenty-three experimental rats showed mild portal cirrhosis, compared with 16 control rats. Sixteen experimental rats showed mild bile-duct proliferation compared with 7 control rats. One experimental rat had a well-developed cholangioma and 2 control rats had hepatomas. Thus, while it would seem that there was rather more cirrhosis and bile-duct proliferation in the treated group, these changes were also present in the control group. Two-thirds

of the rats in both experimental and control groups showed hyperplasia and hyperkeratosis of the forestomach epithelium ; one-third of both groups had keratinising squamous papillomas of the forestomach.

(e) 2-*Naphthylamine* by spoon to rabbits : Three male and 3 female rabbits were fed on a mixed diet of oats, hay and mangels or green food according to the season. Twice per week 200 mg. of powdered 2-naphthylamine was given orally by means of a spoon. The animals maintained their weight satisfactorily and were killed when it appeared that they would not survive longer (Table V). The epithelial changes in the bladder were of a minor order. In Rabbit 12 there was a tiny transitional-cell benign papilloma at $4\frac{3}{4}$ years (Fig. 19) ; in Rabbit 8 there was epithelial hyperplasia with early downgrowth simulating an adenoma at $5\frac{1}{4}$ years.

EXPLANATION OF PLATES.

FIG. 5.—Bladder of Mouse 82, 2-amino-1-naphthol hydrochloride pellet for 39 weeks. The bladder is viewed from above, after cutting away the dome. The dilated urethral orifice is seen as a dark circle above the centre. Immediately adjacent are 2 small papillomata. To the left and right are projecting tumours. × 4.

FIG. 6.—Bladder wall of Mouse 84, 2-amino-1-naphthol hydrochloride pellet for $4\frac{1}{2}$ weeks, showing simple hyperplasia. × 35.

FIG. 7.—Bladder wall of Mouse 119, paraffin wax pellet for 21 weeks, showing urethral orifice. There is marked epithelial hyperplasia, accentuated by tangential cutting. This is the most advanced change seen in any mouse of this group. × 30.

FIG. 8.—Bladder of Mouse 85, ammonium chloride in paraffin wax pellet for 37 weeks. Wall nearly normal. × 6.

FIG. 9.—Bladder of Mouse 91, 2-naphthylamine pellet for 34 weeks. Wall nearly normal. The dilated ureter is seen at bottom right. × 6.

FIG. 10.—Bladder wall of Mouse 96, 2-naphthylamine pellet for 33 weeks. Localised patch of epithelial hyperplasia close to dilated urethral orifice. × 45.

FIG. 11.—Bladder wall of Mouse 76, 2-amino-1-naphthol hydrochloride (synthetic) pellet for 34 weeks. Squamous metaplasia on left and mucous metaplasia on right. The mucus stained red with muci-carmine. × 60.

FIG. 12.—Bladder wall of Mouse 109, 2-amino-1-naphthol hydrochloride (*in vivo*) pellet for 30 weeks. Adenoma with commencing downgrowth but no invasion of muscle. × 130.

FIG. 13.—Bladder wall of Mouse 81, 2-amino-1-naphthol hydrochloride (synthetic) pellet for 38 weeks. Transitional-cell papilloma, just invading muscle on left. × 60.

FIG. 14.—Bladder wall of Mouse 82, 2-amino-1-naphthol hydrochloride (synthetic) pellet for 39 weeks. Transitional-cell carcinoma with downgrowth in central portion. × 20.

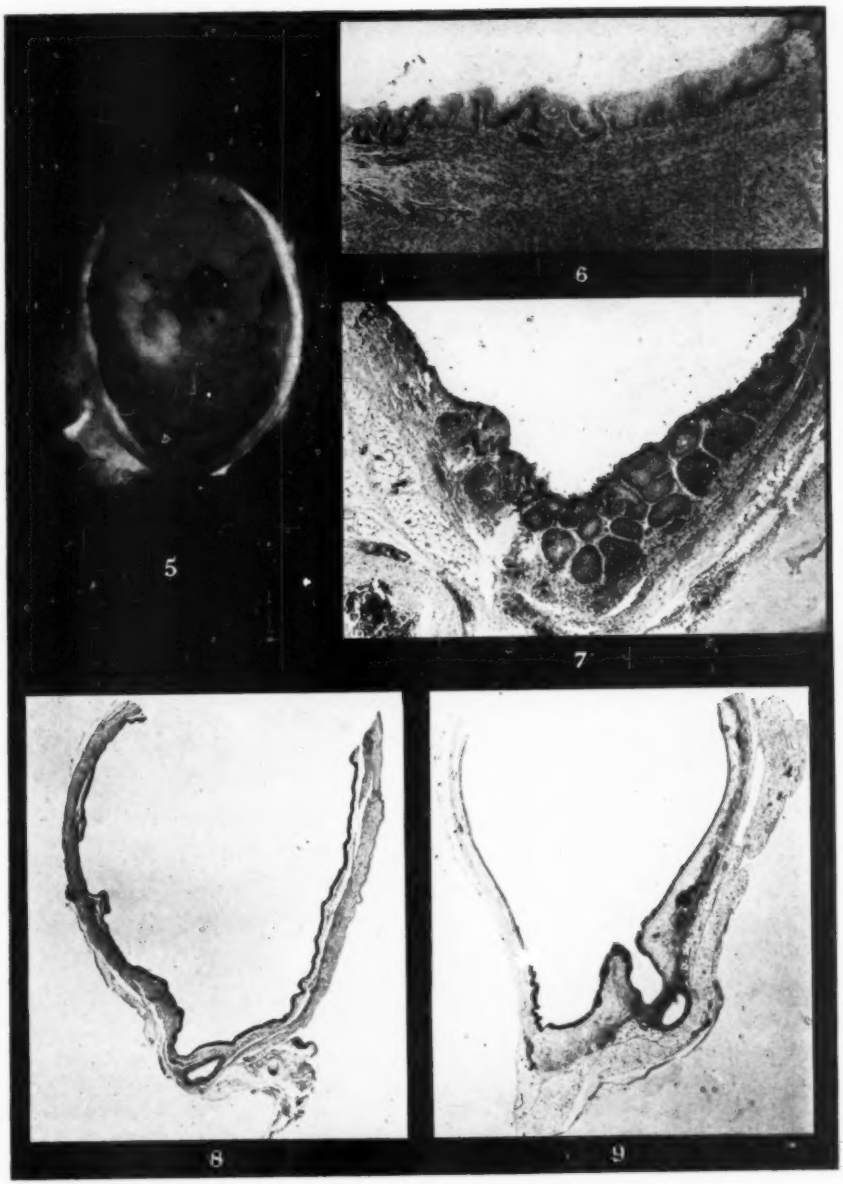
FIG. 15.—Bladder wall of Mouse 24, 2-amino-1-naphthol hydrochloride (synthetic) pellet for 31 weeks. Transitional-cell carcinoma invading the muscular wall of the bladder as far as the epithelium of the dilated ureter. A narrow band of muscle still remains on the right. × 50.

FIG. 16.—Bladder wall of Mouse 126, 3:4:5:6-dibenzcarbazole pellet for 14 weeks. Transitional cell papilloma. × 50.

FIG. 17.—Bladder of Mouse 175, 3:4:5:6-dibenzcarbazole pellet for 17 weeks. The lower half of the bladder is filled with cancer, while the upper part is partially contracted. Some suture material is visible top right. The uterus is seen posterior to the bladder. × 6.

FIG. 18.—IF female mouse, receiving 2-naphthylamine by stomach-tube for 72 weeks. Multiple cholangiomatous areas. × 85.

FIG. 19.—Male Rabbit 12, fed with 2-naphthylamine for $4\frac{3}{4}$ years. Transitional cell papilloma at the dome, with slight hyperplasia of adjacent epithelium. × 30.



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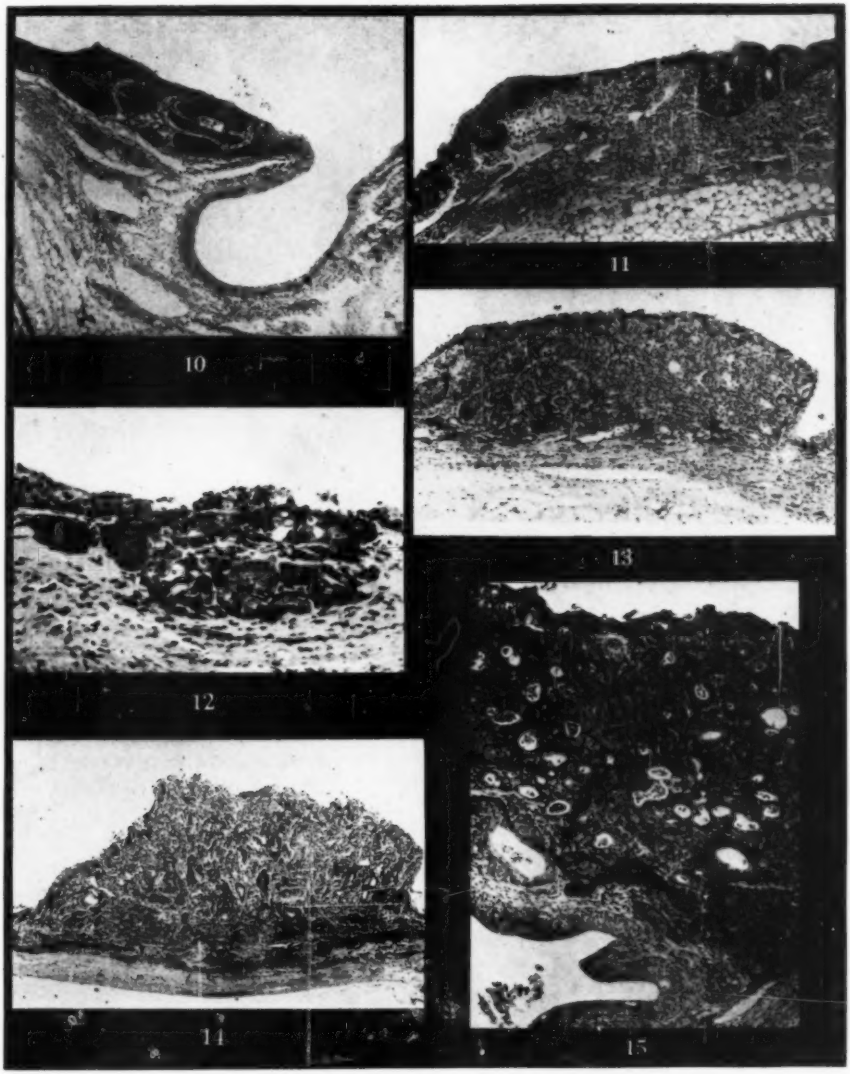

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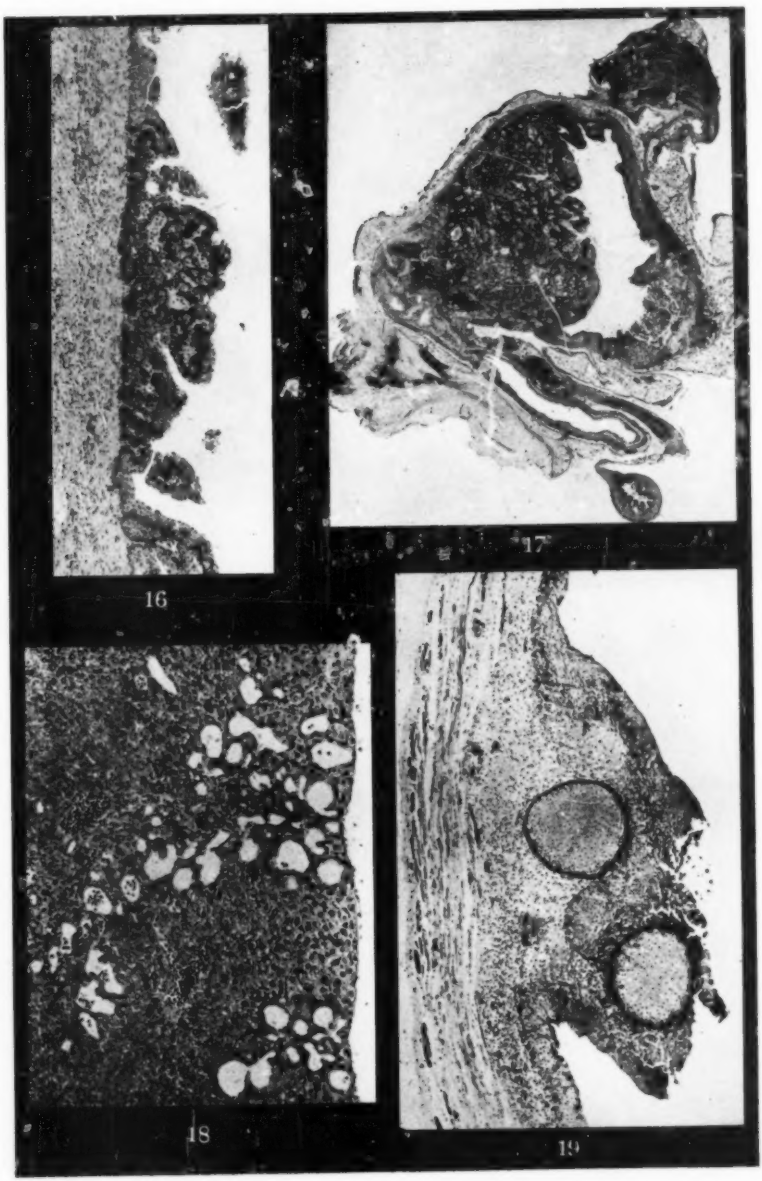

Bonser, Clayson, Jull and Pyrah.

TABLE IV.—*Feeding of Rats with 2-Naphthylamine in Synthetic Diets in which the Protein Content was varied (approx. 310 mg. per kg. body-weight per week).*

Diet + 2-naphthylamine.	Total rats.			Weeks of treatment.								Total bladder papillomas.
	F.	M.	Unknown.	20–29.	30–39.	40–49.	50–59.	60–69.	70–79.	80–89.	Over 90.	
High protein (20 per cent)	10	6	1	0/2	0/2	0/1	0/4	0/2	0/0	0/0	0/6	0/17
Mid protein (10 per cent)	9	5	1	0/1	0/1	0/2	0/0	1/6	0/3	0/0	2/2	3/15
Low protein (5 per cent)	10	7	1	0/0	0/0	0/5	0/1	1/8	0/0	0/0	0/4	1/18
Diet alone.												
High protein (20 per cent)	13	3	1	0/2	0/1	0/0	0/0	0/3	0/1	0/3	0/7	0/17
Mid protein (10 per cent)	5	12	0	0/1	0/7	0/3	0/1	0/2	0/0	0/0	0/3	0/17
Low protein (5 per cent)	7	5	3	0/3	0/5	0/2	0/0	0/1	0/3	0/0	0/1	0/15

Numerator = number of rats with bladder papillomas. Denominator = number of rats dying within the period stated.

TABLE V.—*Oral Administration of 2-Naphthylamine to Rabbits = 100 mg. per kg. body-weight per week.*

No.	Colour.	Sex.	Period of treatment (years).	Approximate total dose of chemical administered (g.)	Changes in bladder.
7	Black	Female	2¾	58	Normal.
9	„	„	4	84	„
12	Chinchilla	Male	4¾	100	Transitional cell papilloma.
11	„	Female	5¼	110	Normal.
10	„	Male	5¼	110	„
8	Wild	„	5¼	110	Epithelial hyperplasia with downgrowth.

Experiment 3.

Subcutaneous injection of mice and rats.

(a) 2-*Amino*-1-*naphthol hydrochloride* in RIII and stock mice : A fresh suspension of the synthetic chemical was made in arachis oil, and each mouse was injected subcutaneously in the flank once per fortnight with 5 mg. per 100 g. body weight. Great difficulty was experienced in finding the maximum tolerated dose and even this amount caused ulceration of the skin on occasion. Twenty-three mice survived for 40 weeks and 3 subcutaneous spindle-cell sarcomas were observed at the site of injection (Table VI). Other lesions such as hepatoma and leukaemia were observed, but these conditions have also been seen in untreated mice and are not necessarily attributable to the treatment. The bladders were normal.

(b) 2-*Amino*-1-*naphthol hydrochloride* in albino rats, derived from the Glaxo stock : Subcutaneous injections were made as in the mice. Fourteen rats survived to the tumour period and 5 subcutaneous spindle-cell sarcomas were observed at the site of injection (Table VI). All these tumours invaded underlying voluntary muscle and one invaded also the pleural and peritoneal cavities. There were metastases in the lungs from one tumour.

TABLE VI.—*Subcutaneous Injection of Mice and Rats with 2-Amino-1-naphthol Hydrochloride in Arachis Oil (5 mg. per 100 gm. body-weight per fortnight.)*

Animal.	No. M.	No. F.	Weeks of treatment.	Type of lesion.	Survival (weeks). 40–49.	50–59.	60–69.	over 70.	Total. incidence.
Stock mice	6	9	65	Subcutaneous sarcoma	1/0	1/3	0/5	0/7	2
				Hepatoma	0/0	1/3	3/5	0/7	4
				Leukaemia	0/0	0/3	3/5	0/7	3
RIII mice	4	4	29	Subcutaneous sarcoma	0/3	1/1	0/2	0/2	1
				Leukaemia	0/3	0/1	1/2	0/2	1
Stock rats	8	6	Until death	Subcutaneous sarcoma	1/1	3/4	1/1	0*/8	5

* = 6 rats still alive after 80 weeks of treatment.

Numerator = number of animals showing the lesion. Denominator = number of animals dying within the period stated.

Experiment 4.

Painting of mice.

2-Naphthylamine in IF mice : A saturated solution of the chemical in benzene was painted on the skin between the shoulder-blades once per week. In spite of a satisfactory survival rate (Table VII) no tumours of the skin nor liver occurred.

TABLE VII.—*Painting of Skin of IF Mice with Saturated Solution of 2-Naphthylamine in Benzene.*

Total mice.		Weeks of treatment.			Tumours of skin.	Tumours of liver.
F.	M.	40–49.	50–59.	60–99.		
9	16	7	3	15	0	0

DISCUSSION.

There is no certain knowledge of the mode of entry of 2-naphthylamine into workers in the chemical industry. Three routes may be involved—ingestion, inhalation, and absorption through the skin. Therefore, after the discovery by Hueper and his colleagues in 1938 (Hueper, 1938 ; Hueper, Wiley and Wolfe, 1938) that bladder cancer could be induced in dogs by feeding the chemical, this method was chosen for testing smaller domestic animals with a view to avoiding the expense and other difficulties inherent in treating dogs. When it had been shown by Bonser, Clayson and Jull (1951) that the metabolite of 2-naphthylamine present in greatest quantity in the urine of the dog after feeding was 2-amino-1-naphthyl sulphuric acid (II), it was necessary to reconsider the mode of exhibition of the various substances in the light of their chemical properties. 2-Naphthylamine base is a relatively stable compound, is oil and benzene-soluble, and can be used for feeding, injection or painting. 2-Amino-1-naphthol hydrochloride is an unstable compound, which is easily oxidised, and is water-soluble. Moreover, from the results of metabolism and biological experiments in various species (Bonser, Clayson and Jull, 1951) it seemed probable that to act as a carcinogen it would require to be present at a certain level of concentration. This contention would be greatly strengthened should the cat prove to be a susceptible animal.

It was for these reasons that the idea of introducing the compound incorporated in a solid vehicle into the lumen of the mouse bladder was explored (Jull, 1951). It was postulated that a high diffusion rate could be expected, that conditions inside the bladder would tend to be anaerobic, and that the exposure of the epithelium to the carcinogen would be almost continuous. In addition, intermediary metabolism by the liver would be eliminated.

The results of Experiment 1 are regarded as sound evidence that 2-amino-1-naphthol hydrochloride is a local carcinogen, whereas 2-naphthylamine is not. This statement is supported by the fact that bladders implanted with paraffin wax remained free from tumours, whereas in bladders implanted with the known carcinogens 20-methylcholanthrene (Jull, 1951) and 3:4:5:6-dibenzcarbazole tumours occurred well within the experimental period of the negative results (Fig. 1 to 4). The induction of tumours by the local action of the metabolite of 2-naphthylamine shows also that there is no factor inherent in the mouse bladder which precludes the development of tumours in this form of carcinogenesis.

Additional evidence for the carcinogenic activity of 2-amino-1-naphthol hydrochloride has been obtained. A few local sarcomas have occurred in mice and rats

following subcutaneous injections in oil (Table VI). Those obtained in mice would seem to have significance, as although many subcutaneous injections of the same oil containing oestrogens and other chemicals have been made into mice in this laboratory, no sarcomas have hitherto been observed. The 5 sarcomas in rats are probably of less significance, as the subcutaneous tissues of this species are so singularly reactive to foreign materials of any kind. In 1938 Hueper obtained 3 retothelial sarcomas in mice by intraperitoneal injection of impure 2-amino-1-naphthol in stock mice (Hueper, 1938).

By contrast, no evidence has yet been brought forward to suggest that pure 2-naphthylamine is locally carcinogenic, though our own results of subcutaneous injection in oily solution into mice are not yet ready. The few sarcomas induced by Hackmann (1951) followed the use of the impure technical compound. 2-Naphthylamine does, however, induce distant tumours in mice, in the form of hepatomas in the CBA and cholangiomas in the IF strains. It is suggested that the metabolite is acting locally on the liver cells. Local tumours have not been observed following the feeding of dogs, mice, rats and rabbits. Nor did Rhoads (quoted by Hartwell, 1951), in a large experiment, find tumours elsewhere than in the bladder in the rat. No tumours occurred in mice after painting a benzene solution on the skin (Table VI). In general, aromatic amines are not locally carcinogenic, though there are notable exceptions, such as 2-anthramine (Bielschowsky, 1946), and the amino-stilbenes (Haddow, Harris, Kon and Roe, 1948).

Taking all the above evidence into consideration the case is very strong for accepting 2-amino-1-naphthol or its conjugates as the active carcinogen formed by the metabolism of 2-naphthylamine in man and the dog. It seems worthy of emphasis that such a conclusion would scarcely have been possible without the positive evidence obtained by the direct introduction of the test substances into the mouse bladder. This experiment indicates that it is the metabolite which is carcinogenic and not an impurity present in the 2-naphthylamine, as was suggested many years ago and has recently been re-emphasised by Case and Pearson (1952). The 2-naphthylamine used for the implantations was from the same batch as that used for the feeding and painting experiments. The 2-amino-1-naphthol was synthesised in this laboratory by sodium dithionate reduction of 2-nitroso-1-naphthol, and it seems unlikely that either 2:2-dinaphthylamine or 3:4:5:6-dibenzcarbazole and pyrene, suggested as impurities in 2-naphthylamine by Case and Pearson (1952), would be present in this substance. Moreover, when 2 per cent 20-methylcholanthrene in paraffin wax was implanted into the mouse bladder (Jull, 1951) no tumours were obtained in 4 mice surviving from 21 to 42 weeks. This concentration of carcinogen is very much greater than that suggested for the possible impurities of 2-naphthylamine.

There is no evidence to differentiate between the direct action of 2-amino-1-naphthyl sulphuric acid on the bladder epithelium or its further chemical transformation in the bladder. Such chemical transformation might take the form of hydrolysis to give 2-amino-1-naphthol or the formation of polycyclic or other molecules.

An important observation in both the industrial and experimental diseases is the location of the tumours in the bladder and not in the rest of the urinary tract. The rate of excretion of a dose of 2-naphthylamine administered orally to the dog was shown to reach a peak approximately 4 hours later, (unpublished observation). Thus it is probable that urine containing an effective level of the meta-

bolite passes down the renal tubules, pelvis and ureter for a period of about 4 hours, whereas urine stored in the bladder before, during and after the peak may contain an effective level for a longer period. Some other mechanism may also be involved, as when 2-amino-fluorene was fed to rabbits, ureteric as well as vesical tumours were obtained (Bonser and Green, 1950).

2-Amino-1-naphthol is an ortho-hydroxyamine and the hypothesis is being examined that other carcinogenic aromatic amines are active by virtue of their conversion in the body to compounds of this type. In preliminary experiments, using an impure sample of 1-amino-2-naphthol hydrochloride, supplied by The British Drug Houses Ltd., evidence of carcinogenic activity has been obtained in 5 out of 6 mice by the bladder implantation method (unpublished observation). Walpole, Williams and Roberts (1952) are pursuing similar ideas in the 4-aminodiphenyl series.

The evidence which has been brought forward here to show that a metabolite of 2-naphthylamine is carcinogenic supports the case for a more vigorous pursuit of metabolites from other chemicals which may be taken into the human body. The types of substance which immediately come to mind are the aromatic amines and some of those chemicals which are so liberally used as food-colouring matters at the present time. Further, knowledge about the metabolic changes which take place in other groups of chemicals, while not having immediate practical application as far as industry is concerned, might enable an opinion to be expressed as to whether it would be safe to introduce new types of chemical into industrial or other processes before the damage is actually done.

SUMMARY.

Preliminary experiments had suggested that 2-amino-1-naphthol hydrochloride was a local carcinogen when introduced directly into the lumen of the mouse bladder (Bonser, Clayson and Jull, 1951). Further experiments have shown that this method is practicable on a larger scale. Metaplasia, papilloma and carcinoma were induced when 2-amino-1-naphthol hydrochloride and 3:4:5:6-dibenzcarbazole were tested, but no tumours were obtained with 2-naphthylamine nor paraffin wax (Fig. 1 to 4).

Additional evidence of the carcinogenic properties of 2-amino-1-naphthol hydrochloride was obtained by the occurrence of a few sarcomas in mice and rats following subcutaneous injection in oil (Table VI).

No local tumours occurred when 2-naphthylamine was administered orally to mice, rats and rabbits. Hepatomas occurred in mice, which suggested that the metabolite was acting locally on the liver cells. A few benign bladder tumours occurred in rats and rabbits at a late date in the experiments (Tables IV and V).

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THE STORAGE OF AVIAN LYMPHOID TUMOUR STRAINS AT LOW TEMPERATURES.

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STRAINS of transplantable avian lymphoid tumours have been isolated from natural cases of avian lymphomatosis by Pentimalli (1940), Olson (1941), Burmester and Prickett (1945).

Three of these strains, RPL 12, 16 and 19, isolated by Olson (1941), Burmester and Prickett (1945) and Burmester and Denington (1947) respectively, have been the subject of study at this laboratory. The cytological features of these strains have been well described by the above-mentioned workers, but, in brief, the type cell is larger than that normally found in lymphoid tissue. The tumour cells are uniform in size and appearance ; the nucleus is vesicular with one or two nucleoli. The cytoplasm of these cells forms a narrow crescent around the nucleus and stains an intense blue with methods employing methylene blue.

Following the subcutaneous inoculation of tumour material from these strains a local tumour results, with frequent metastasis to the viscera (Burmester and Prickett, 1945 ; Burmester, 1947 ; and Davis, 1952). In our first transmission experiments with these strains, 90 to 95 per cent of chicks inoculated were killed after an average interval of 8 to 10 days (Darcel, 1952).

Burmester, Brandly and Prickett (1944) and Burmester (1950) showed that the tumour material from these strains retained its tumour-producing activity when stored *en masse* at temperatures below — 40° C. This property is of major importance, since it obviates the necessity of serial passage with its attendant disadvantages of the number of experimental animals required, the labour involved and the danger of introducing other infectious agents, during passage from chick to chick.

The storage of tumour material in the form of suspensions rather than *en masse* has certain advantages in experimental oncology (Craigie, 1949*b*). If conditions can be devised whereby such suspensions retain a certain degree of activity over a long period, it should be possible to use material in experiments with a predetermined tumour-producing activity.

In the experiments to be described it will be shown that these lymphoid tumour suspensions can retain tumour-producing activity at these low temperatures in a similar manner to the C_3H sarcoma (Craigie, 1949*b*). Craigie (1949*a* and 1949*b*) found that the suspending fluid may have a considerable influence on the activity of stored material, the incorporation of dextrose in the suspending fluid exerting a protective action. In these experiments the possibility that dextrose might act in a similar way on these avian lymphoid tumour strains was investigated and confirmed.

In the experiments to be described the mean time for first appearance of tumours following inoculation has been used, as an index of the relative activity of the material before and after freezing. This has followed our confirmation of the observations of Gottschalk (1943, 1946) and Craigie (1949*a*) that the interval before tumours appear after inoculation regularly increases with progressively smaller inocula. In our experiments the latent period was found to be subject to errors associated with individual differences in response and the subjective element involved in the detection of tumours. The possible heterogeneity of slopes of regressions of the response on the dose, determined in experiments in which groups of chicks were inoculated with varying amounts of tumour material prepared from different donors, suggested limitations in the use of the latent period in comparing the activity of material prepared from different chicks. In spite of this it was felt to be a useful criterion of activity where differences were large. On the average a ten-fold dilution increased the latent period by approximately 2 days. The number of tumours, on the other hand, did not closely reflect differences in activity, since in experiments in which large numbers of chicks were inoculated with the same dose of tumour material, in spite of the significant differences in the latent period and survival time observed, the number of takes seldom fell significantly below 100 per cent. Considerable dilution of tumour suspensions was necessary before any reduction in the frequency of tumours was observed (Darcel, 1952).

EXPERIMENTAL METHODS.

Suspensions were prepared with the aid of a special tumour grinder (Craigie, 1949*c*) from pectoral tumours or tumour involved livers. The suspending fluids used have been physiological saline or 6 and 10 per cent aqueous dextrose solutions. Where pectoral tumour was used the coarseness of the mince necessitated its filtration by gravity through a A.G. 74XO sintered glass filter.

Three types of refrigeration at temperatures below — 70° C. were used :

(*a*) An electric refrigerator maintained at a temperature of — 70° C. (subsequently referred to as Refrigerator A).

(*b*) An insulated box maintained at a temperature of below — 70° C. with CO_2 ice (subsequently referred to as Refrigerator B).

(*c*) A double vacuum flask containing crushed CO_2 ice in the small inner compartment (1 quart capacity) and larger outer flask (1 gallon capacity). Ampoules to be stored were placed in the inner flask. The whole unit was placed in a refrigerator running at — 25° C. (subsequently referred to as Refrigerator C).

In these investigations whilst ampoules of other sizes have been used, thin-walled sterile 3 ml. glass ampoules sealed by heat after the suspension has been pipetted into them have now been adopted as the standard.

The chicks used were Barred Rock × Brown Leghorns. Each chick has been inoculated in the first week of life with 0·25 ml. of the material, the activity of which was being tested subcutaneously into the pectoral region. The chicks have been palpated daily to determine the mean time of the first appearance of tumours at the site of inoculation, which was used as an index of the activity of the tumour material.

All chicks dying or killed at the end of the observation period of 3 to 4 weeks

were autopsied for the presence or absence of tumours. Chicks dying from causes unassociated with the tumour inoculations have not been included in these records.

In some experiments tumour suspensions were divided into two, one lot being stored at a temperature below — 70° C. and the other at — 25° C.

In these experiments suspensions were tested for activity before freezing and after storage in ampoules at low temperatures, by inoculating them into groups of chicks, usually 4 or 5, as indicated in Table I.

EXPERIMENTAL OBSERVATIONS.

The results of these experiments are shown in Table I. Three out of the four saline suspensions stored in A, one of the six stored in B and the only one stored in C produced tumours when inoculated into chicks. On the other hand, five out of the six dextrose suspensions stored in A for comparable periods produced tumours. The only dextrose suspension stored in B and both stored in C produced tumours. Altogether four out of ten saline suspensions and eight out of nine dextrose suspensions produced tumours.

Suspensions of tumour material from batches T337, T348, T358, T428 and T484 prepared with dextrose showed greater activity than corresponding material suspended in saline, as evidenced by shorter latent periods for the development of tumours, or by the failure of the latter but not the former to develop tumours.

Part of one batch of material T428 was stored in ampoules as tumour mince without diluting. After storage and subsequent thawing, it was diluted with saline to give approximately the same concentration as a 1/5 suspension. This, when inoculated in terms of mean time of appearance of tumours, appeared to be intermediate in activity between the corresponding material stored in saline and that stored in dextrose.

Some of the suspensions were stored for considerable periods before testing for activity. Of 4 lots of ampoules stored for more than 100 days in A, 3 showed the ability to produce tumours when inoculated into susceptible chicks.

The ampoules containing saline and dextrose suspensions stored at — 25° C. showed no tumour-producing activity after storage, even in cases where the same suspension was still active after storage at — 70° C.

DISCUSSION.

The factors determining the survival of cells in suspension at low temperatures are complicated and have been discussed in detail by Craigie (1952) and Lasnitzski (1952). This series of experiments has confirmed for those tumours the protective effect of dextrose first demonstrated by Craigie (1949*a*, 1949*b*), when used for suspending tumour cells stored at low temperatures. The mechanism by which dextrose exerts its protective effect is still largely a matter for speculation ; Craigie (1949*b*) suggested that in part its action might be associated with its influence on electrolyte or ion distribution. Craigie (1949*b*) also suggested that the development of even more effective suspending fluids might lead to the satisfactory preservation of tumour-producing activity with commercially available equipment operating at temperatures as high as — 20° C. to — 18° C. Unfortunately, in our experiments, whilst dextrose exerted a protective action at — 70° C., this action was not demonstrable at — 25° C.

TABLE I.—*The Effect of Storage at Low Temperatures on the Activity of Tumour Suspensions.*

Tumour material.					Refrigeration.		Results of inoculation.			
Batch No. T—.	Strain.	Source.	Suspending fluid.	Concentration of suspesnion.	Method.	Time stored in days.	Number of chicks inoculated.	Total number of tumours developing.	Number of tumours palpated.	Mean time in days for palpable tumour to appear.
335/327	19	Liver	(*a*) Saline	1/10	A	0	6	6	5	7·20 ± 0·02
			(*b*) „	1/10		147*	4	0	0	
			(*c*) 10% dextrose	1/10		147*	4	0	0	
337/804	12	„	(*a*) Saline	1/10	A	0	5	5	5	5·40 ± 0·6
			(*b*) „	1/10		135*	3	2	2	14·00 ± 0
			(*c*) 10% dextrose	1/10		135*	3	3	3	7·00 ± 1·40
348/901	16	„	(*a*) Saline	1/10	A	0	40	40	37	4·32 ± 0·09
			(*b*) „	1/10		121*	2	2	2	8·00 ± 0
			(*c*) 10% dextrose	1/10		121*	4	4	4	7.50 ± 0·25
358/P	16	Pectoral tumour	(*a*) Saline	1/5	A	0	5	5	5	4·00 ± 0
			(*b*) „	1/5		108	3	3	3	10·00 ± 0·58
			(*c*) 6% dextrose	1/5		108	5	5	5	8·60 ± 0·24
382/14	16	Liver	(*a*) Saline	1/10	B	0	5	5	4	5·00 ± 0
			(*b*) „	1/10		29*	5	0	0	
			(*c*) „	1/10		35	5	0	0	
411/136	19	„	(*a*) „	1/10	B	0	5	5	3	6·00 ± 0
			(*b*) „	1/10		24*	2	0	0	
411/120	19	„	(*a*) „	1/10	B	0	5	5	4	6·00 ± 0
			(*b*) „	1/10		25*	8	0	0	
			(*c*) „	1/10		35	5	0	0	
414/712	12	„	(*a*) „	1/10	B	0	4	4	4	6·00 ± 0
			(*b*) „	1/10		24	5	0	0	
420/P	12	Pectoral tumour	(*a*) 6% dextrose	1/5	A	0	8	8	7	5·14 ± 0·40
			(*b*) 6% „	1/5		17	7	7	7	7·43 ± 0·48
		Liver	(*c*) 6% „	1/5		17	9	9	9	7·11 ± 0·44
428/407	19	Pectoral tumour	(*a*) Saline	1/5	B	0	8	8	8	6·13 ± 0·23
			(*b*) „	1/5		12	5	5	4	17·50 ± 0·50
			(*c*) 10% dextrose	1/5		0	7	7	6	6·14 ± 0·26
			(*d*) 10% „	1/5		12	5	5	5	10·66 ± 0·77
			(*e*) Tumour mince	—		12	7	7	7	11·43 ± 0·20
484/604	16	Liver	(*a*) Saline	1/10	C	0	4	4	4	6·50 ± 0·71
			(*b*) „	1/10		9	3	0	0	
			(*c*) 6% dextrose	1/10		0	5	5	5	6·60 ± 0·30
			(*d*) 6% „	1/10		9	4	4	4	11·00 ± 0·97
533/9019	16	Pectoral tumour	(*a*) 6% „	1/10	C	0	8	8	8	5·50 ± 0·18
			(*b*) 6% „	1/10		3	6	6	6	7·33 ± 0·33

* Ampoule of suspension also stored at − 25° C., but inactive when tested.

It has been suggested that the rate of freezing and thawing might considerably affect the activity of stored material (Lépine, Barski and Reinié, 1951). Burmester (1950) presented evidence that these factors did affect the activity of the transplantable avian lymphoid tumours stored *en masse*, since material frozen slowly retained its activity after storage, whereas corresponding material frozen rapidly was devoid of activity after storage. Gye, Begg, Mann and Craigie (1949) failed to observe any effect of variations in the rate of freezing on the activity of tumours stored at low temperatures. In these present experiments no attention has been given to the rate of freezing and thawing. The same authors (Gye, Begg, Mann and Craigie, 1949) showed that at room temperature, material stored at a low temperature rapidly lost its tumour-producing activity. Smith and Parkes (1951) also observed that ovarian tissue stored by freezing at these low temperatures deteriorates rapidly in its ability to produce successful implants, unless implanted immediately. This may explain the few successful storage experiments with CO_2 ice-box refrigeration (B), since circumstances prevented stored tumour suspension from being inoculated until $1\frac{1}{2}$ to 2 hours after its removal from the ice box.

Burmester (1950) observed that certain lots of tumour material failed to retain its activity during storage *en masse*. This is also true of these present experiments, where, as shown in Table I, several ampoules containing suspensions prepared with saline in the case of T335/327, T382/14, T411/136, T411/120, T414/712, T484/604 and one suspension in 10 per cent dextrose T335/327 failed to produce tumours. This appeared to be unrelated to the activity of the material stored.

If further experiments with these lymphoid tumour strains show that, after the initial drop of activity regularly observed in these experiments, further losses during storage are limited, the utilisation of tumour material or predetermined activity would be a considerable asset in many types of experiments with these tumours.

The demonstration that tumours may successfully be stored in double vacuum flasks containing CO_2 ice should prove of considerable value to workers without access to the more expensive forms of refrigeration.

SUMMARY AND CONCLUSIONS.

1. Suspensions of avian lymphoid tumour material in physiological saline or dextrose solutions retain their tumour-producing activity when stored at temperatures below — 70° C., but not when stored at — 25° C.

2. Dextrose solutions at 6 and 10 per cent concentrations in distilled water exerted a protective effect as compared with physiological saline on tumour-producing activity of suspensions after storage at — 70° C. No protective action of dextrose was demonstrated at — 25° C.

3. Tumour suspensions retained tumour-producing activity in all three types of low temperature refrigeration employed, i.e., (*a*) an electric refrigerator running at temperatures down to — 125° C., (*b*) an insulated box containing blocks of CO_2 ice, and (*c*), a simple double vacuum flask containing CO_2 ice in outer and inner compartments.

4. Certain lots of material stored at these low temperatures were inactive when tested. In all the remaining ampoules tested there was a partial loss in tumour-producing activity. The reasons for the loss in activity have not been investigated.

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CYTOLOGICAL RESULTS OF GROWING TISSUES UNDER AN OIL FILM.

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For investigating the action of fat-soluble substances on living cells *in vitro*, it is usual to apply them, either as suspensoid colloids, or dissolved in the oil phase of emulsions. Ludford (1934*b*) found that fat-soluble coloured compounds, such as Sudan III and Sudan Black, dissolved in serum, and in this form could be used for vitally staining fat droplets in cells. This suggested that another possible way of treating cells with a fat-soluble substance might be to dissolve it in oil and then apply small drops to the surface of explants of cover-slip cultures, for presumably diffusion would then occur into the serum of the culture medium. To investigate this possibility Sudan Black was dissolved in medicinal liquid paraffin, and a small drop was deposited with a syringe on each explant of a number of cultures of fibroblasts and sarcoma cells. In due course fat droplets in all types of cells became coloured. However, certain changes were observed in the cells which continued to grow under the oil films. It was therefore considered desirable that these should be studied before further experimental work was carried out with chemical compounds dissolved in oil.

EXPERIMENTAL PROCEDURE AND RESULTS.

Cover-slip cultures were used throughout. The culture medium consisted of equal parts of chick embryo or rat spleen extract, and a mixture of fowl plasma and either rat serum or rat plasma. Liquid paraffin was applied to the explants after two or three days' incubation by means of a syringe fitted with a very fine needle. The oil spread over the explants and extended as a thin film over the growing cells. Before fixing cultures they were washed repeatedly with warm Ringer solution. Thorough washing to remove all oil was essential in order to obtain good staining. The fixatives employed included Susa, Alcohol-Bouin and Helly, and staining was carried out with various preparations of carmine and haematoxylin. Observations were confined to primary cultures.

Morphological changes in fibroblasts.

Fibroblasts emerge from explants of mouse embryo heart as elongated spindle-shaped cells (Fig. 1). Twenty-four hours after the application of a drop of liquid paraffin to the surface of a two-, or three-days-old culture the cells are spread out laterally (Fig. 2). With continuation of growth the peripheral cells become separated, and disperse in the surrounding medium (Fig. 3). They flatten until they are little more than fine films of protoplasm. In old cultures some cells

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become greatly enlarged and resemble the giant cells of tumours. Also, areas of cytoplasm are to be seen which contain several nuclei, usually of varying sizes, between which it is impossible to discern any dividing membranes (Fig. 4). Presumably in such instances the fine films of protoplasm, which previously surrounded each nucleus, have coalesced. A comparable fusion of cells occurs in some tumours and has been designated " plasmogamy " (Howard and Schultz, 1911).

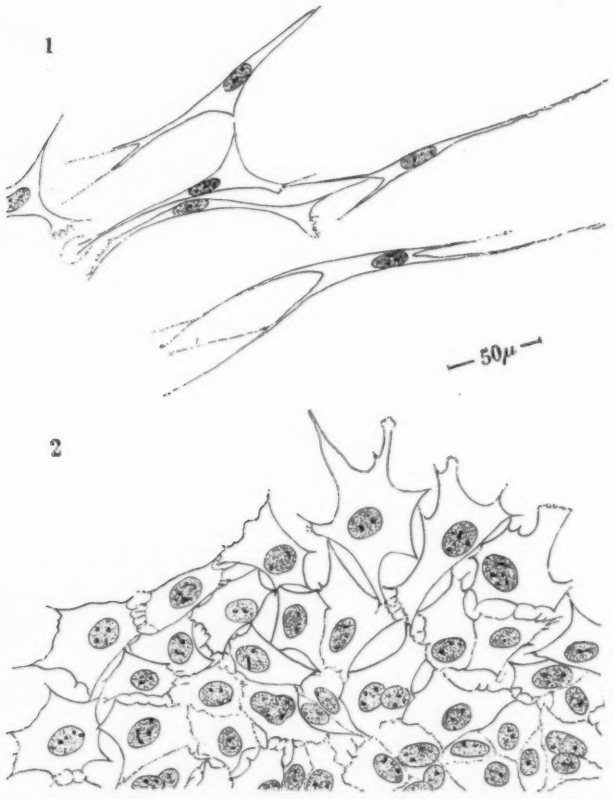


FIG. 1.—Fibroblasts from the periphery of a 5-days-old control culture.
FIG. 2.—Part of the periphery of a 3-days-old culture of fibroblasts, 24 hours after covering with liquid paraffin.

Another effect of the oil is to accelerate the outgrowth of fibroblasts from explants, so that after two days well-spread sheets of cells are obtained. Provided that the oil is completely washed off before fixing cultures, their subsequent staining is greatly facilitated because the plasma which has been in contact with the oil does not stain so intensely as that of the controls. Thus it is possible to obtain preparations of extensively spread cells, well stained and without

appreciable coloration of the surrounding medium, which obviously facilitates cytological study.

Mitosis in fibroblasts.

The flattening of fibroblasts induced by the oil does not prevent nuclear division, but cytoplasmic cleavage is made difficult. Typically, a cell at the onset of mitosis becomes rounded, but this rarely happens in treated cultures. Mitosis occurs throughout the outgrowths, even in isolated peripheral cells.

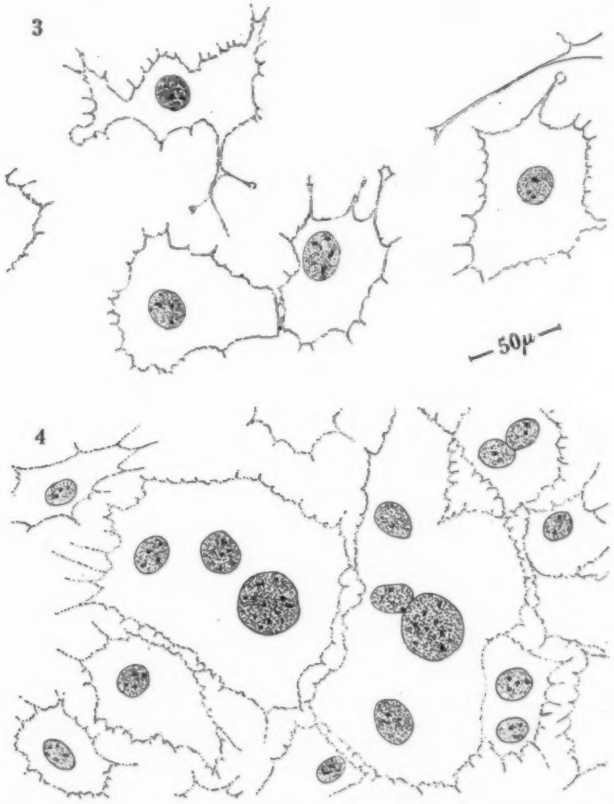


FIG. 3.—Flattened fibroblasts dispersed around the periphery of a 9-days-old culture, covered with liquid paraffin 6 days.

FIG. 4.—Fusion of enlarged extensively spread fibroblasts from the inner zone of growth of the same culture as depicted in Fig. 3.

Fig. 6 depicts cells on the edge of a culture, and Fig. 7 was photographed from a part of an outgrowth where the cells were separating. It is not uncommon to find two adjacent cells at the same phase of mitosis. This occurrence is illustrated in Fig. 7, where there are two early telophases, and in Fig. 8 where there

are two late telophases. The most feasible explanation is that the two cells in each figure are the daughter cells of a preceding division, and that owing to their identical constitution, and the same environmental conditions, they grew synchronously and began to divide at approximately the same time.

Despite the flattened state of the cells the formation of the chromosomes and their separation into two groups occurs in the normal manner. Obviously, the spindle mechanism is not impaired.

The action of the oil is revealed at the end phase of mitosis. Normally, when cleavage begins the peripheral cytoplasm is involved in a " bubbling " process, which continues as the two daughter cells separate. This is illustrated in Fig. 5, which depicts a dividing cell from a control culture photographed at a higher magnification than Fig. 6 and 7. In the cultures treated with oil there are seen various transition stages between cells exhibiting almost the normal amount of "bubbling ", and its complete absence in the most spread cells. The latter fail to divide, and give rise to binucleate cells. Dividing cells with small peripheral bubbles are often seen, and it would seem that complete separation into two daughter cells may occur while the cytoplasm is considerably spread out. Of the flattened cells shown in Fig. 8 the upper one has completed division, except for a connecting strand of protoplasm, but it is doubtful whether the lower one would have done so. The result of nuclear division without cytoplasmic cleavage is seen in Fig. 9. The two nuclei, which have not yet completed the structural organization of the interphase, have drawn apart in the well-spread cytoplasm. Sometimes cells at this phase elongate and although the nuclei are widely separated (Fig. 6), they have not been observed to complete division. Later the two nuclei come together, as in the cell on the right-hand side of the same figure. Occasionally the two nuclei of such cells are seen to enter simultaneously the prophase of another mitosis. Should this be completed the result, of course, will be a cell with double the normal number of chromosomes. To what extent such occurrences rather than endomitosis are responsible for the polyploid cells of cultures is problematical.

DESCRIPTION OF PLATES.

FIG. 5.—Late telophase from a control culture at higher magnification, showing peripheral " bubbling " of the cytoplasm.

FIG. 6.—Mitosis in fibroblasts on the margin of an outgrowth from an explant of mouse embryo heart. Five days' growth : under liquid paraffin 2 days.

FIG. 7.—Two telophases in mouse fibroblasts grown 4 days under liquid paraffin. Six-days-old culture.

FIG. 8 and 9.—Aberrations of the late phases of mitosis in mouse fibroblasts grown under liquid paraffin.

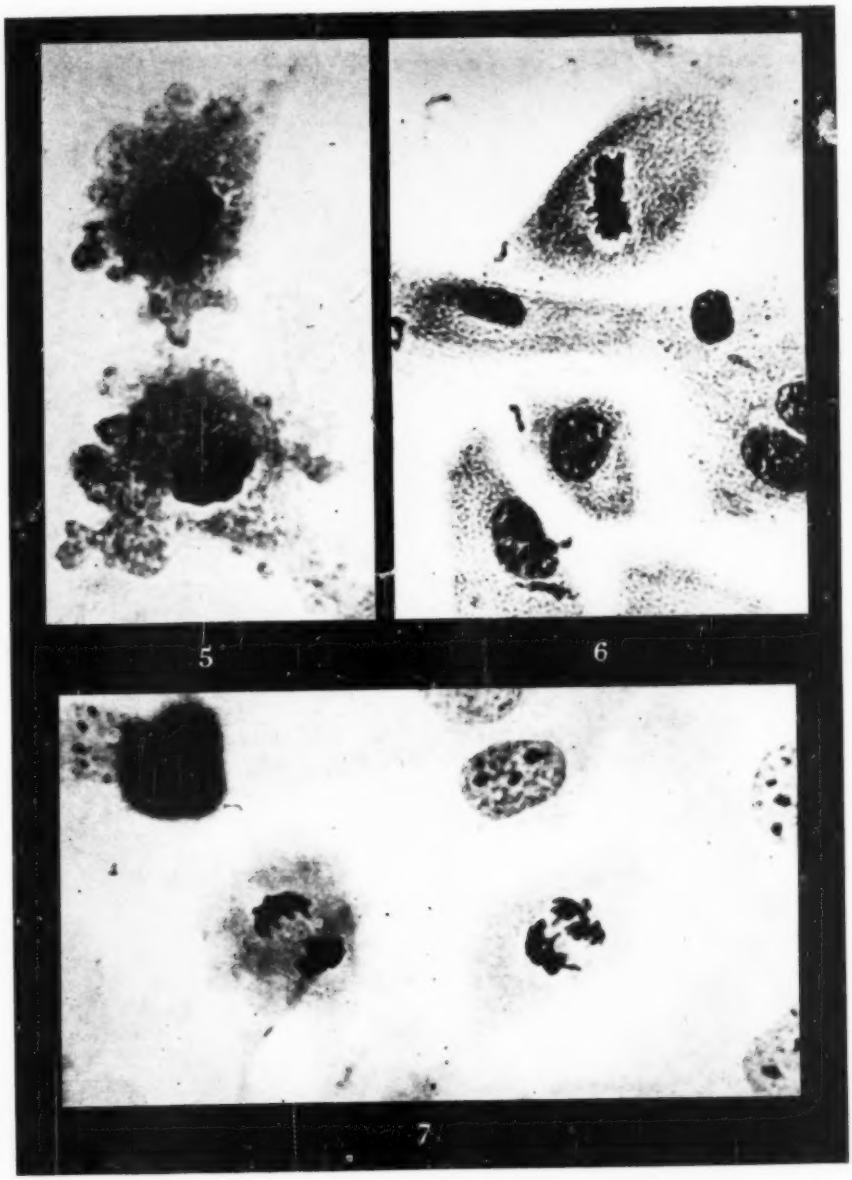
FIG. 8.—Cytoplasmic division almost complete in the top cell ; lower cell failing to divide. Five-days-old culture : under liquid paraffin 3 days.

FIG. 9.—Daughter nuclei at late stage of reconstruction, without cytoplasmic cleavage. Six-days-old culture : under liquid paraffin 4 days.

FIG. 10 and 11.—Malignant cells of the Crocker sarcoma.

FIG. 10.—Part of the periphery of a 6-days-old culture where the sarcoma cells are most spread.

FIG. 11.—More extensively spread cells from the margin of a 6-days-old culture covered with liquid paraffin 4 days.



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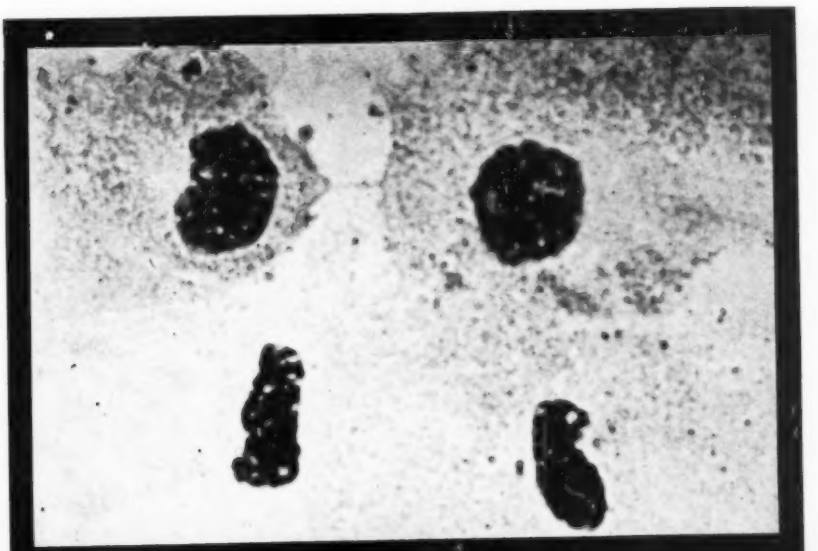

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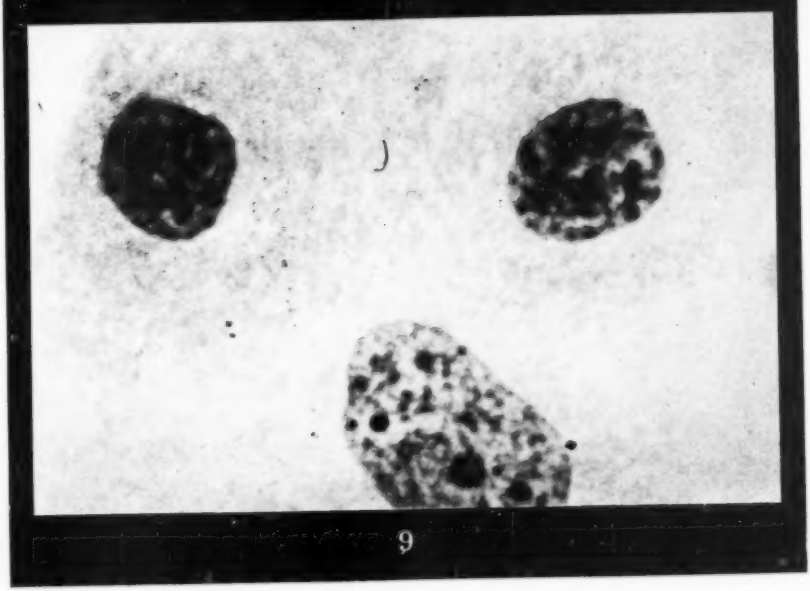

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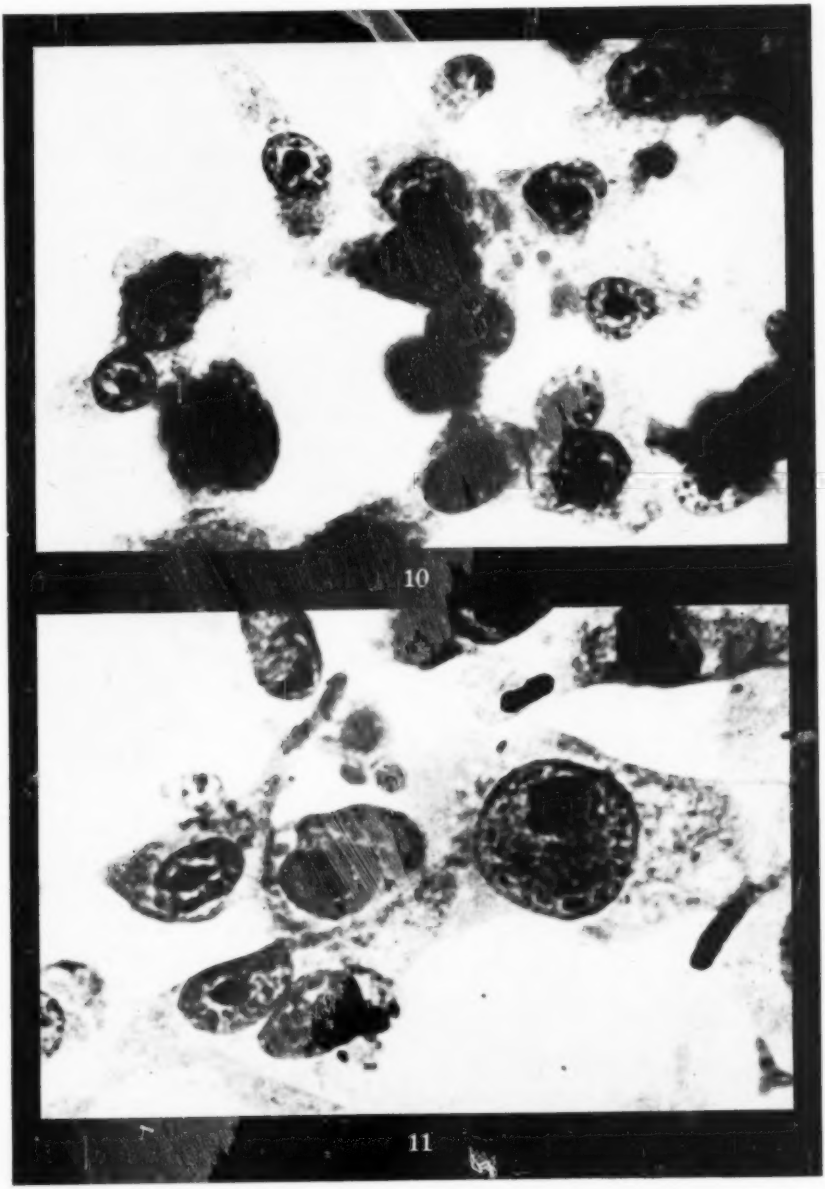

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11

Hypertonicity of the culture medium induced by partial drying causes flattened cells to become rounded. Under such conditions a binucleate cell may divide directly so that its nuclei become separated in two round daughter cells.

Since fibroblasts tend to spread out, even in control cultures, especially in old ones, similar aberrations of telophase to those described above can often be detected. Indubitably they are the primary cause of the binucleate cells often seen in tissue cultures.

Morphological changes in sarcoma cells.

The ability to spread out on a glass surface in a fluid culture medium varies with the malignant cells of different sarcomata (Ludford, 1934*a*, 1939). The Crocker sarcoma is one of the tumours whose cellular behaviour has been studied previously. Malignant cells removed from a tumour growing rapidly in the animal body, and explanted in a fluid medium, form a cluster of spherical cells. The difference in the behaviour of such cells and fibroblasts has been attributed to an alteration in the submicroscopic structure of the protoplasm of the malignant cells. It therefore seemed desirable to investigate whether the cells of this sarcoma would behave in the same manner as fibroblasts when covered with an oil film.

In Fig. 10 are shown malignant cells from the margin of a 6-days-old control culture of the Crocker sarcoma photographed from an area where the cells were most spread. The action of the oil is illustrated in Fig. 11. The cells are more spread than those of the control, but not to the same extent as are fibroblasts under the same conditions. In older cultures the sarcoma cells separate and are dispersed in the surrounding medium as happens in fibroblast cultures.

The most striking difference between fibroblasts and Crocker sarcoma cells is the failure of the latter to divide when flattened. No phases of mitosis are discernible in the peripheral outgrowth of the sarcoma cells, when there are innumerable mitoses in the unflattened cells immediately surrounding the explants.

DISCUSSION.

This study of the effect of an oil film on cells growing *in vitro* has demonstrated the considerable morphological changes which fibroblasts can undergo. Tumour cells have been more intensively studied than the cells of healthy tissues, and special significance has often been attributed to the polymorphism of malignant cells. Nevertheless, the same property is exhibited to a lesser extent by normal cells, and differences become less obvious when both types of cells are grown under identical conditions in tissue cultures.

The aberrations of telophase in flattened fibroblasts are such as might reasonably be anticipated in view of experimental work carried out with fertilised eggs of invertebrates. Danielli (1952) has recently confirmed the observations of Zeuthen (1951) that when fertilised eggs are flattened between two plates nuclear division is uninterrupted, but cleavage is inhibited so that multinucleate cells originate. He found further that a small glass plate resting on fertilised eggs was raised at the onset of mitosis, but on its completion was lowered again. He suggests that " immediately before division there is an increase in the tension at the cell surface, and that in the plane which was occupied by the metaphase chromosomes this tension reaches a higher value than elsewhere, so that cleavage ensues in this plane ". On the assumption that a similar process is involved in

the division of fibroblasts the aberrations of telophase could be due to the oil lowering the surface tension of the cells. A similar action on interphase cells would explain their spreading. Why Crocker sarcoma cells when flattened should fail to enter mitosis as fibroblasts do under exactly similar conditions remains completely obscure.

SUMMARY.

The application of a thin film of liquid paraffin to the surface of cultures of fibroblasts induces the cells to spread laterally. The peripheral cells become separated, and disperse in the surrounding medium. Considerable enlargement of cells occurs in old cultures, where cellular fusion results in multinucleate cells. Flattened fibroblasts complete nuclear division in the usual manner, but cytoplasmic cleavage is abnormal, or fails to take place so that binucleate cells are formed. Malignant cells of the Crocker sarcoma under similar conditions flatten to a lesser extent, and when they do so no mitosis occurs.

I am indebted to my colleague Mr. G. Calcutt, B.Sc., for the photomicrographs illustrating this paper.

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